NUTRITIONAL STUDIES WITH THE DUCK

III. NIACIN DEFICIENCY [1]

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ONE FIGURE

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In previous reports the performance of young ducklings fed simplified diets (Hegsted and Stare, '45) and a diet deficient in pyridoxine (Hegsted and Rao, '45) has been described. The rapid growth of the duckling compared to its relatively small initial weight apparently makes storage of nutrients a minor factor in the body economy. Those deficiencies which have been studied to date develop with surprising rapidity and often are apparent within 3 or 4 days after the animals are fed the deficient diet.

In this paper are reported the results of studies made with niacin deficient diets.

EXPERIMENTAL

The ration used had the following percentage composition: sucrose 49.7, SMA casein 18, gelatin 10, corn oil 10, salt mixture 5, mono-calcium phosphate 1, cod liver oil 2, liver fraction "L" [2] 4 and choline 0.3. Crystalline vitamins [3] with the exception of nicotinic acid were added at the levels used in

[1] Supported in part by a grant-in-aid from the John and Mary R. Markle Foundation.

[2] Supplied by the Wilson Laboratories, Chicago, Ill.

[3] Supplied by Merck and Co., Rahway, N. J.

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previous work (Hegsted and Rao, '45) and alpha tocopherol, 50 mg per kilo, and menadione, 1 mg per kilo, were mixed with the ration. The ration was essentially the same as previously described, although the corn oil content had been increased by 6%.

Day-old ducklings were purchased and fed a commercial mash for 3 or 4 days before they were given the experimental diet. Their average weight when the experiments were started was from 60 to 70 gm.

RESULTS

The average weight of ducklings which received the ration containing increasing amounts of nicotinic acid is shown in table 1. It is seen that great differences in growth were apparent even on the fifth day of the experiment. The total gain of the birds on the purified basal diet was only 40 gm in 20

TABLE 1

The growth response to nicotinic acid of ducklings fed various diets.

DIET	NO. OF DUCKS	NIC. ACID ADDED	NIC. ACID CONTENT (CALCULATED)	AVERAGE WEIGHT			
				5 days	10 days	15 days	20 days
		mg/ 100 gm	*mg/ 100 gm*	*gm*	*gm*	*gm*	*gm*
Purified	10	0	.6	83	87	94	100
Purified	4	0.5	1.1	88	117	146	176
Purified	14	1.0	1.6	114	163	219	318
Purified	7	2.0	2.6	145	271	406	590
Purified	7	3.0	3.6	130	280	399	550
Goldberger	4	0	1.0[2]	86	114	130	145
Goldberger	4	5.0	6.0	115	175	282	440
Goldberger + 4% "L"[1]	4	0	1.6	89	124	192	260
Goldberger + 4% "L"[1]	4	5.0	6.6	117	194	297	465

[1] Wilson liver extract fraction "L".

[2] Calculated from the figure of 1.43 mg of nicotinic acid per 100 gm of yellow corn meal (Committee on Food Composition of the Food and Nutrition Board, Tables of Food Composition, Washington, 1943).

Fig. 1 Ducklings 3 weeks of age fed the simplified diet. The bird on the left received the diet with no added nicotinic acid.

days, compared to approximately 500 gm for those receiving 2 or 3 mg of nicotinic acid per 100 gm of ration.

The minimum requirement appears to be approximately 2 mg of nicotinic acid plus that contained in the ration. According to the figures published by Cannon et al. ('45) the casein should supply only 0.00648 mg per 100 gm of ration while the liver extract supplied 0.64 mg.[4] The requirement is thus about 2.5 mg per 100 gm of ration.

[4] The figure for the nicotinic acid content of the liver extract was kindly supplied by S. W. Heir, Wilson Laboratories, Chicago, Ill.

The relatively high nicotinic acid requirement is also easily demonstrated by the use of a modified Goldberger ration. This ration contained 71% yellow corn, 18% casein, 4% salts, 5% cottonseed oil, and 2% cod liver oil. Vitamins were added at the same levels used in the simplified ration. The growth on this ration with and without nicotinic acid and liver extract is also shown in table 1. After supplementation with all of the known crystalline vitamins, a diet composed chiefly of corn is severely deficient in nicotinic acid for the duck.

The symptoms of deficiency on both the purified and the Goldberger rations are not striking. The ducks are small, weak (fig. 1), and have diarrhea. The cloaca usually appears large and full of fluid. The eyelids are often stuck together, which may be due in part to the stickiness of the high sugar rations since the condition appears to be more severe on the purified diet. In some of the birds there appeared to be an accumulation of food underneath the tongue and some necrotic tissue underneath this. However, nothing comparable to the black tongue (Briggs et al., '42) or the dermatitis (Briggs et al., '43) which have been described in nicotinic acid deficient chicks was seen.

DISCUSSION

The performance of ducklings fed the Goldberger ration is to be contrasted with that of chicks since Waisman and Elvehjem ('40) were unable to demonstrate a clear-cut response to nicotinic acid in chicks fed this diet and various combinations of the other vitamins. The dog, on the other hand, appears to be more comparable to the duck. For both of these species the Goldberger diet is primarily lacking in nicotinic acid. Whether these differences in response are referable to the low tryptophane content of the corn diet and species differences in the ability to substitute tryptophane for nicotinic acid remains to be investigated. The dog and the chick both develop an inflammation of the tongue and mouth parts while this has not been noted in deficient ducklings. It may be recalled, however, that Handler and Dann

('42) found that under certain conditions black tongue was often not a symptom of dogs dying of nicotinic acid deficiency. Thus the mouth symptoms may be related to factors not obvious at the present time.

Recently Briggs ('46) has reported that the symptoms of nicotinic acid deficiency in the turkey poult are comparable to those found in chicks. Perosis is also reported as a symptom in these two species but was not seen consistently enough in ducklings to be considered the result of nicotinic acid deficiency.

The development of a nicotinic acid deficiency on diets consisting primarily of corn may suggest a consideration of this vitamin in the practical feeding of ducks. It may also be noted that this diet is low in factors supplied by the liver extract, presumably the L. casei factor (Hegsted and Stare, '45).

SUMMARY

Young ducklings rapidly develop a nicotinic acid deficiency on purified diets low in nicotinic acid as well as on the modified Goldberger diet containing large amounts of corn. The symptoms observed were lack of growth, diarrhea, and general weakness.

The requirement for rapid growth on the purified diet is approximately 2.5 mg per 100 gm of ration.

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NUTRITIONAL STUDIES WITH THE DUCK

IV. THE EFFECT OF VITAMIN DEFICIENCIES ON THE COURSE OF P. LOPHURAE INFECTION IN THE DUCK AND THE CHICK [1]

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Only few experimental data are available on the influence of specific nutritional deficiencies upon the course of malaria. Passmore and Sommerville ('40) placed monkeys on a diet similar to that of the rice-eating poor of India. This diet is suboptimum in at least vitamins A and C and in calcium. The course of malaria (P. cynomolgi and P. knowlesi) in the deficient animals did not appear to be different from that in the controls. Trager ('43) produced biotin deficiency in ducks and chicks by feeding them a diet containing a large proportion of dried egg white and infected them with P. lophurae. For both species of hosts, the average peak parasite number was 50% to 100% higher in the deficient animals than in the controls; the highest peak was always reached in a deficient animal and the lowest in a control; and more of the deficient animals died of the infection. Uninfected controls very rarely died of biotin deficiency. It was shown that the increased parasitemia was not a general effect of malnutrition since pantothenic acid deficient birds were not more susceptible than corresponding controls.

Seeler, Ott and Gundel ('44) confirmed the finding of Trager with regard to biotin deficiency and have extended the studies

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to several other deficiency diseases. Riboflavin deficiency (Seeler and Ott, '44) causes the infection produced by P. lophurae to be less severe as judged by the degree of parasitemia. On the other hand, protein deficiency (Seeler and Ott, '45a) and "folic acid" deficiency (Seeler and Ott, '45b) each caused a more severe infection with considerably higher degrees of parasitemia.

In the present report the effects of deficiencies of nicotinic acid, thiamine, choline, and vitamin A upon the course of P. lophurae infections in ducks and chicks are presented. As is well known, the normal chick is fairly resistant to this infection. Death seldom occurs unless the inoculum is extremely large. The duckling, however, is very susceptible, and in our experience only an occasional bird survives the disease. We have thus compared the response to the same parasite of a resistant and a susceptible species fed identical diets. As shown previously (Hegsted and Stare, '45), the nutritional requirements of chicks and ducks appear qualitatively similar.

METHODS

Pekin ducklings and white Leghorn chicks were used. The animals were 1 to 7 days old when received from the hatchery. They were housed in wire mesh cages and in most of the experiments given free access to duck pellets and chick mash, respectively, until they had reached a weight of 90 to 130 gm. In a few studies the birds were given the experimental diet when 2 or 3 days old. The animals were then divided into groups of three to nine of comparable weight and put on the experimental diet ad libitum. A simplified ration developed in this laboratory (Hegsted and Stare, '45) was used for both ducks and chicks. The various deficiencies were produced by simply omitting the appropriate vitamin from the diet. Usually two groups of birds received the diet without the vitamin and two groups the complete diet shown in table 1. In some experiments, two additional groups of animals were fed diets containing intermediate levels of the vitamin.

Before infection with malaria, the animals were weighed daily; thereafter, every other day. When the deficiency had become evident in the group on the lowest vitamin level, one group at each vitamin level was infected with malaria, its companion serving as a noninfected control. A decrease in the rate of growth is usually the first evidence of deficiency,

TABLE 1

Composition of the purified diet.[1]

	PER KILO
Sucrose	492 gm
Casein, SMA, "vitamin-free"	180 gm
Gelatin	100 gm
Salts IV[2]	50 gm
$CaHPO_4$	10 gm
Liver extract[3]	40 gm
Corn oil	100 gm
Cod liver oil[4]	20 gm
Choline chloride	3 gm
Biotin, 100 mg per ml in 50% alcohol	2 ml
Mixture of water-soluble vitamins[5]	10 ml
Mixture of fat-soluble vitamins[6]	5 ml

[1] The crystalline vitamins were supplied by Merck and Co., Inc., Rahway, N. J.

[2] Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart, J. Biol. Chem., vol. 138, p. 459, 1941.

[3] Wilson's Liver Fraction "L" — supplied by the Wilson Laboratories, Chicago, Ill.

[4] For the experiments on vitamin A deficiency, no cod liver oil was added; 120 gm of corn oil and 10,000 I.U. of viosterol were used per kilo of ration. As a source of vitamin A, a concentrated fish liver oil containing 100,000 I.U. per gm was used. This oil contained very little vitamin D.

[5] Containing thiamine hydrochloride, 100 mg; calcium pantothenate, 400 mg; riboflavin, 200 mg; nicotinic acid, 1000 mg; pyridoxine hydrochloride, 100 mg in 250 ml of 95% alcohol.

[6] Containing alpha tocopherol, 1000 mg; menadione, 10 mg in 100 ml corn oil.

and the birds were inoculated 1 to 3 days after this was evident. In studies on vitamin A and thiamine deficiency, it was necessary to supply small amounts of these vitamins in the diet to prevent the animals from dying before the studies were complete. All deficiencies occurred much earlier in the duck than in the chick. This can probably be ascribed to the much more rapid growth of the former animal.

The size of the inoculum in the experiments on ducks varied from 2×10^5 to 10^8 parasites per kilo of body weight, the same amount being used on all animals in the same experiment. Chicks were inoculated with 5×10^8 parasites per kilo of body weight. The parasitized blood was obtained from donor ducks. It was diluted with 0.8% sodium chloride, so that the dose to be administered was contained in 0.2 to 1.0 ml and immediately injected intravenously into the animals. Daily smears were taken, starting 2 or 3 days after inoculation. They were stained with Wright's stain and 500 red blood cells were counted to permit the determination of the percentage of parasitized cells.

RESULTS

Nicotinic acid

Deficiency of this vitamin in chicks manifested itself after 7 days in a slight retardation of growth. The mortality due to the deficiency alone was very low, probably because this diet contains approximately 0.6 mg nicotinic acid per 100 gm (Hegsted, '46). The mortality in both deficient and non-deficient chicks infected with malaria was also low.

The absence of nicotinic acid in the diet has a pronounced effect upon the course of malaria. In the first experiment (table 2), the peak of the parasitemia in deficient chicks was four times as high as that in the controls (42.5% as compared with 11.1%) and occurred 2 days earlier. In the animals that received one-sixth of the full amount of nicotinic acid, the peak was 18.1% and coincided in time with that in the non-deficient group. Almost all animals were able to get rid of their infection by the thirteenth day after inoculation. It is of interest that the deficient group, in spite of the much more severe infection, became free of parasites in approximately the same number of days after infection as the controls. Re-infection with 16×10^8 parasites per kilo did not produce a parasitemia.

A second experiment in which the degree of infection was somewhat less in both groups confirmed the increased sus-

TABLE 2

Average weight and percentage of parasitized red cells of nicotinic acid deficient chicks as compared with nondeficient animals.[1]

DAYS	NO NICOTINIC ACID			NICOTINIC ACID 0.5 MG %		NICOTINIC ACID 3.0 MG %		
	Group 1	Group 2		Group 3		Group 4	Group 5	
	Weight	Weight	% Parasites	Weight	% Parasites	Weight	Weight	% Parasites
0	114(8)[2]	105(9)		95(9)		104(9)	118(7)	
7	160	137	Inoculated	151	Inoculated	162	175	Inoculated
10		150	9.7	163	0.9	175	191	0.2
11	172		12.1		1.8			0.6
12		155	39.6	177	4.7	182	202	1.1
13	181		42.5		13.1			3.8
14		152	36.0	185	14.2	195	211	5.9
15			28.7		18.1			11.1
16		157	16.2	185	14.1		223	10.4
17	200	160(8)	9.0	197(8)	8.2	219	230(6)	2.8
19	217	165	1.8	212	3.3	242	246	0.0
21	229	167	0.0	223	6.8	260	254	0.0
23	244	176	0.1 (Reinoculated)	246	0.8 (Reinoculated)	272	287	0.0 (Reinoculated)
28		187		270			357	
29			0.0		0.0			0.0
33			0.0		0.0			0.0

[1] Groups 2, 3, and 5 were inoculated with 5×10^5 parasites per kilo; groups 1 and 4 served as controls. The animals were 18 days old at the beginning of the experiment.

[2] Figures in parenthesis = number of animals.

ceptibility of nicotinic acid deficient chicks (table 3). The parasitemia of the controls reached a peak of 3.2% on the sixth day, of the deficient animals 16.6% on the seventh day. Both groups became free of parasites on the eleventh day after infection.

In similar experiments using groups of six to eight ducklings, a consistent difference was not found between the parasitemia of the deficient animals, as compared with that of the controls. Typical results are indicated in table 4. In two

other comparable experiments, the deficient birds appeared slightly more susceptible in one but somewhat less susceptible in the second.

TABLE 3

Average weight and percentage of parasitized red cells of nicotinic acid deficient chicks as compared with nondeficient animals.[1]

DAYS	NO NICOTINIC ACID			3 MG PER CENT NICOTINIC ACID		
	Group 1	Group 2		Group 3	Group 4	
	Weight	Weight	% Parasites	Weight	Weight	% Parasites
0	104(5)[2]	103(8)		104(5)	104(7)	
3	128	119		122	124	
7	143	135		144	144	
8	146	142	Inoculated	161	162	Inoculated
10	152	147	0.1	181	178	0.1
12	179(4)	152(7)	2.9	201	186	1.6
14	180	143	2.2	222	194	1.5
15			2.9			1.8
16			11.1			3.2
17	191	169(4)	16.6	250	212	2.3
18			13.0			1.0
19	206	166	5.7	292	221	0.2
21	220	185	0.0	311	221	0.4

[1] Groups 2 and 4 were inoculated with 5×10^6 parasites per kilo; groups 1 and 3 served as controls. The animals were 19 days old at the beginning of the experiment.

[2] Figures in parenthesis = number of animals.

Thiamine

The absence of thiamine in the diet of chicks did not appear to influence the course of malaria to a significant degree. The peak of the parasitemia occurred in the twelfth day after inoculation and was 21.7% for the deficient birds, 19.2% for birds receiving the diet plus 80 μg of thiamine per 100 gm of diet, and 21.4% for birds on the complete diet. Severe deficiency symptoms occurred in the first group and 75% of the birds died on the thirteenth day. Growth was suboptimum in the partially deficient group.

Ducks given a ration containing 80 μg of thiamine per 100 gm began to show weakness of the legs and difficulty in walking after 6 days. This amount of the vitamin was apparently sufficient to prevent convulsive seizures. The animals continued to gain weight, though at a decreased rate as compared with the nondeficient controls. A thiamine deficiency of this extent did not significantly modify the course of malaria. The

TABLE 4

Average weight and precentage of parasitized red cells of nicotinic acid deficient ducks as compared with nondeficient controls.[1]

DAYS	NO NICOTINIC ACID			3 MG PER CENT NICOTINIC ACID		
	Group 1	Group 2		Group 3	Group 4	
	Weight	Weight	% Parasites	Weight	Weight	% Parasites
0	105(4)[2]	108(8)		113(2)	108(6)	
2	118	117	Inoculated	150	153	Inoculated
4	122	132	0.3	220	210	6.3
5			2.3			1.8
6	124	137	4.9	292	266	2.8
7	135	138	18.7	340	275	10.2
8			39.2			31.9
9	131	140	41.6	401	288	42.2
10	125	135(7)	31.4	466	286	31.0
11			15.3			12.6
12	132	141(6)	11.6	552	322(3)	0.5

[1] Groups 2 and 4 were inoculated with 10^8 parasites per kilo; groups 1 and 3 served as controls. The animals were 8 days old at the beginning of the experiment.

[2] Figures in parenthesis = number of animals.

peak of the number of parasitized red cells in the deficient group amounted to 41.6% and was reached 11 days after inoculation. In the control group a peak of 50.8% was reached on the same day. All but one of the eight nondeficient ducks died of the infection within the period of observation (13 days), whereas four out of eight deficient animals were alive at the end of the experiment.

Choline

Inconsistent results have been obtained with choline deficient chicks. In two experiments some of the deficient chicks appeared highly susceptible and the deficient groups as a whole were found to have a significantly higher parasitemia than the controls. In two later experiments the same wide variation was found but statistical treatment failed to indicate any difference in susceptibility between the two groups. Differences in the degree of deficiency may account for these differences.

Ducks placed on a ration without added choline showed a retardation of growth after 3 days. After another 5 days, the first deficiency manifestations appeared in the form of weakness of the legs. These symptoms rapidly progressed to typical perosis so that eventually the animals were unable to stand or walk, though they continued to gain weight at a slow rate. Within the 18 days of observation only one out of five ducks died of the deficiency. The course of malaria appeared somewhat milder in the deficient animals; a statistically significant difference was found to exist between the two groups of eight ducks 6 days after inoculation (15.9% parasitemia in the deficient ducks, 32.6% parasitemia in the controls), but the difference was less marked in the succeeding days.

Vitamin A

Chicks on the purified diet without vitamin A showed a normal rate of growth during the first 14 days, as compared with the controls (table 5). A moderate retardation of growth became evident after this period. Nine days later the animals began to lose weight and most of them died in the course of the next 4 days. After 21 days on the deficient diet, the chicks began to show weakness of the legs and ataxia. These symptoms rapidly progressed, so that finally the animals were unable to stand or reach their food. Convulsions were not seen.

Only a few of the nondeficient chicks died of malaria within the period of observation (8 days). The infection ran a milder

course in the deficient animals; the parasite count was consistently lower than in the controls as shown in table 5.

In the ducks placed on a low vitamin A diet, symptoms of the deficiency appeared after 8 days and almost all animals died within 4 days after the first appearance of symptoms. In the

TABLE 5

Average weight and percentage of parasitized red cells of vitamin A deficient chicks as compared with nondeficient animals.[1]

DAYS	NO VITAMIN A			10,000 I.U. VITAMIN A PER KILO		
	Group 1	Group 2		Group 3	Group 4	
	Weight	Weight	% Parasites	Weight	Weight	% Parasites
0	71(6)[2]	70(8)		65(5)	66(7)	
5	99	99		93	94	
10	130	123		127	125	
14	154	157		167	155	
16	164	169		189	170	
18	176	183		215	193	
19	182	184		224	200	
20			Inoculated			Inoculated
21	186[3]	190[3]		254	209(6)	
23	180	167	1.5	267	215	2.2
24			1.7			4.8
25	161	156(7)	3.3		224	14.6
26	(5)	(5)	10.1			33.8
27	154	169(2)	36.2	318	229	49.9
28	(2)		54.0		(5)	51.2
30	(0)	(0)				

[1] Groups 2 and 4 were inoculated with 5×10^6 parasites per kilo; groups 1 and 3 served as controls. The animals were 10 days old at the beginning of the experiment.

[2] Figures in parenthesis = number of animals.

[3] Indicates first evidence of weakness and ataxia of legs which gradually progressed.

studies on malaria, 80 I.U. of vitamin A were added per kilo of ration to keep the birds alive.

No differences were found in the course of malaria of the deficient animals as compared with the controls. In the former, the peak of parasitemia was reached on the seventh day

(76.6%). All ducks had died on the next day. In the controls, the peak (76.1%) occurred on the same day.

DISCUSSION

It is well known that there are marked differences in avian and human malaria, and these need not be repeated here. Nevertheless, the value of work such as that reported in this paper lies in the possibility that it may suggest factors worthy of investigation with human infections. The findings of Seeler and Ott that protein deficiency (Seeler and Ott, '45a) and folic acid deficiency (Seeler and Ott, '45b) and the present demonstration that nicotinic acid deficiency results in more severe infections in avian malaria may be of importance with regard to human malaria. Diets low in protein and nicotinic acid are known to be common in many malarious regions. Recent data indicate that many types of macrocytic anemias respond to folic acid (Anonymous, '46) and thus this deficiency may be not uncommon in the tropics. Assuming that the human malarias respond in a similar manner, these deficiencies might well account for the long suspected relationship between famine and malaria.

Those deficiencies which have been found to inhibit the development of the parasite, i.e., vitamin A and riboflavin (Seeler and Ott, '44), are less likely to be of practical importance. The experimental studies have all been done using acutely deficient birds. Such acute deficiencies are probably uncommon in humans. In this regard it is interesting that the nicotinic acid deficiency in chicks reported here was very mild and produced only a slight decrease in growth, although the change in susceptibility was profound.

Choline deficiency in chicks was not found to increase susceptibility to malaria. It must be borne in mind that this deficiency in chicks does not produce fatty livers nor hemorrhagic kidneys as have been reported in mammalian species.

The studies reported in this paper suggest that the duck is much too susceptible to be a satisfactory experimental animal for studies with P. lophurae. No differences were observed

in any of the nutritional deficiencies. It seems likely that the parasite reproduces at near maximum rate in the normal duck and increases in susceptibility, should they occur, would be difficult to detect although Trager ('43) has shown that this is possible. The rapid growth of the animals is also perhaps disadvantageous. As observed in table 4, the birds receiving the adequate ration gained over 100 gm during the course of the infection while the deficient birds gained no weight. Thus there was a marked change in blood volume in the growing birds and although the per cent parasitemia was the same in the two groups, the total multiplication of the parasite was undoubtedly considerably greater in the growing birds. Since the factors underlying the rate of multiplication of the parasite in vivo are unknown, it seems useless to speculate on whether or not the correct evaluation of the studies is the percentage of parasitemia or the total increase in parasites in the bird.

In these studies we have not used starvation or paired-fed controls. As has been pointed out previously (Hegsted et al., '46), starvation and protein deficiencies are in some respects identical since in both instances body tissue cannot be formed or is destroyed. Since protein deficiency (Seeler and Ott, '45) increases the susceptibility to avian malaria, such controls may obscure rather than clarify the results. Seeler and Ott ('44) found in fact in their studies with riboflavin that restriction of food intake had the opposite effect of riboflavin deficiency and increased the severity of the infection. Since various deficiencies, all of which inhibit growth, do not have comparable effects on susceptibility, it appears established that the effects of the nutritional deficiencies in avian malaria are specific.

SUMMARY

The effects of nicotinic acid, thiamine, choline, and vitamin A deficiency on the course of P. lophurae infections in chicks and ducklings are reported.

In chicks, vitamin A deficiency appears to cause a somewhat milder infection and choline deficiency may have the opposite

effect. Thiamine deficiency appeared to have no influence on the infection. Nicotinic acid deficiency resulted in a much more severe infection. The percentage of parasitized cells was from four to five times greater in the deficient birds. Both deficient and nondeficient birds were able to clear the blood stream of parasites in approximately the same length of time.

In ducks none of the deficiencies influenced the course of the infection to a marked degree. The duck is probably too susceptible to this infection to be a satisfactory experimental animal.

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EVALUATION OF PROTEINS BY THE NITROGEN BALANCE METHOD[1]

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With the ultimate objective in mind of designing a nutritionally efficient mixture of amino acids for intravenous use, preliminary feeding experiments with dogs were undertaken with casein and two grades of fibrin (crude and pharmaceutical[3]). In some experiments, casein was fortified in various ways with cysteine and methionine. The minimum amount of each protein or protein-amino acid mixture required to maintain nitrogen balance was determined. For the sake of economy of words, it is suggested that this figure be called the MPN (minimum protein nitrogen) value of proteins or amino acid mixtures.

Melnick and Cowgill ('37) have determined the minimum amounts required to maintain nitrogen balance of serum protein, casein, lactalbumin, and gliadin. Their results were calculated on the basis of percentage of calories contributed by the protein when the animal was receiving a total of 70 cal. per kg per day. When their results were recalculated in the same terms used throughout our work, the MPN value for casein was between 217 and 295 mg of nitrogen per kg per day and the MPN value for lactalbumin was between 175 and

[1] This material was presented at the American Chemical Society Meeting, April 11, 1946, at Atlantic City.

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[3] Armour.

200 mg of lactalbumin nitrogen per kg per day. The differences between their work and ours will be brought out in the discussion.

METHODS

Selection and preparation of animals for experimental use

Female dogs in good nutritional condition were selected. To increase the ease of catheterization, an effort was made to use only females which had littered puppies. The animals were weighed and given a vermifuge.

The dogs were placed on a diet supplying an adequate amount of calories (75 cal./kg/day; see composition of the non-protein diet under diets used) and 100 mg of crude fibrin [3] nitrogen per kg per day. When casein was used instead of fibrin, 160 mg of casein nitrogen per day were given. This amount of fibrin nitrogen or casein nitrogen was not sufficient to maintain nitrogen balance if the dog was accustomed to a high nitrogen kennel ration. Gradually the negative balance was reduced and in approximately 8 weeks the animal was in positive nitrogen balance and remained there indefinitely. This period of low nitrogen feeding serves to deplete the animal's protein stores to a minimum. The plasma proteins fall to a level below normal and then remain fairly constant. In table 1, the plasma protein data are summarized. Animals prepared in this manner were used for determining the MPN values of proteins and of proteins fortified with amino acids. The data shown in tables 2 and 3 were obtained on animals which had first been subjected to this preliminary depletion period.

The reader will no doubt wonder why 100 mg of fibrin nitrogen per kg per day or 160 mg of casein nitrogen per kg per day were arbitrarily chosen for the amounts of protein nitrogen given during the depletion period. In preliminary experiments the amount of fibrin or casein given to a dog was slowly reduced over a period of a number of weeks until the animal went into definite negative balance. The amounts of

100 mg of fibrin N/kg/day or 160 mg of casein N/kg/day were found to be slightly above the minimum amounts required to maintain nitrogen balance. In order to shorten the depletion period, new animals were started on the experiments at these levels of fibrin or casein nitrogen.

TABLE 1

DOG NO.	WEEKS ON LOW PROTEIN DIET	PERCENT BLOOD [1] PLASMA PROTEIN	HISTORY OF PROTEIN NITROGEN INTAKE
17	40	4.5	On casein or fibrin intakes supplying minimum or sub-minimum amounts for N balance
	42.5	4.4	100 mg pharmaceutical fibrin N/kg/day
	46.5	4.5	100 mg pharmaceutical fibrin N/kg/day
18	27	4.8	On fibrin intake supplying minimum or sub-minimum amounts for N balance
	29.5	5.4	100 mg pharmaceutical fibrin N/kg/day
	33.5	4.8	100 mg pharmaceutical fibrin N/kg/day
21	19	4.9	On fibrin intakes supplying minimum or sub-minimum amounts for N balance
	21.5	5.4	100 mg pharmaceutical fibrin N/kg/day
	25.5	5.3	100 mg pharmaceutical fibrin N/kg/day

[1] Plasma protein was determined by the method of Drew, Scudder and Papps ('40).

Analysis of urine and feces

Urine was collected under toluene or xylene. It was kept acid with acetic acid to prevent the loss of ammonia.

The dogs were catheterized on the morning of the first day of each period before food was given. Carmine was added to the food of the first day of each period to mark the feces. In the early experiments, the feces were mixed with water and homogenized in a Waring Blender. The mixtures were easily

resuspended before removing samples for analysis. However, it was found that the homogenized feces could be mixed with the urine, thus reducing the number of analyses by one-half. The accuracy of this procedure was checked against separate analyses of the urine and homogenized feces. For analysis, the macro-Kjeldahl digestion procedure was used. A measured portion of the digest was used in a micro-Kjeldahl distillation apparatus.

Diets used

The non-protein portion of the diet consisted of 73 gm sucrose, 20 gm lard,[4] 3 gm corn oil, 0.5 gm of fish liver oil containing 65,000 U.S.P. units of vitamin A and 13,000 U.S.P. units of vitamin D per gm, 4.0 gm of U.S.P. salt mixture I, 0.2 gm choline chloride, 1.0 gm agar, 0.6 mg thiamine hydrochloride, 0.6 mg riboflavin, 12 mg nicotinamide, 0.4 mg pyridoxine, 1.2 mg calcium pantothenate, and a liver concentrate low in nitrogen but rich in vitamin B_c. A sufficient amount of this mixture was given to supply 75 cal./kg/day. The nitrogen contained in this amount of the non-protein diet represented usually considerably less than 5% of the total nitrogen intake.

The proteins studied were S.M.A. vitamin-free casein, crude blood fibrin,[3] pharmaceutical grade fibrin,[3] and lactalbumin.[5] The amounts of methionine and cysteine used in the fortification of the casein (see table 2) were based on the nitrogen content of the casein rather than on the weight of casein because of the slight variation in the percentage of nitrogen between different lots of this protein.

The kennel weight of each animal was used as the "standard weight" upon which were based the rates of administration of the protein diets and the non-protein supplement. Each day a weighed portion of the protein or protein-amino acid mixture was mixed with the non-protein portion of the diet and fed in the morning. The animals regularly ate all of the food offered each day except for a few dogs when they were re-

[4] Globe Plankinton.
[5] Borden.

ceiving extremely low levels of protein intake. In these few cases the laborious procedure of force-feeding the uneaten food each day was avoided. Instead, the entire protein supplement was mixed with only 25 gm of the non-protein diet. This was eaten in a few minutes. The remaining amount of non-protein diet required to supply 75 or 80 cal./kg/day was then placed in the dish. The next morning any unconsumed food left in the dish was weighed.

The hydrolysates

The partial hydrolysate [6] was prepared by the method of White and Elman ('42). It was concentrated in vacuo to a small volume and lyophilized.

The complete hydrolysate [6] was made from casein by refluxing 4 days with 3.5 N sulfuric acid. After removal of sulfate ion, the hydrolysate was concentrated in vacuo to a small volume and lyophilized. This material was shown to be substantially completely hydrolyzed $\frac{\text{carboxyl nitrogen (ninhydrin)}}{\text{Total N — ammonia nitrogen}} = 0.79$.

Tryptophane determinations on supplements E-120c and E-120a were done by the method of Shaw and McFarlane ('38).[7]

RESULTS

Unfortified crude beef blood fibrin [8] was found to be one and one-half to two times as effective in maintaining nitrogen balance as unfortified casein. Fortification of casein with cysteine resulted in considerable improvement over unfortified casein. This finding paralleled previously reported experience with rat growth experiments by Osborne and Mendel ('15). Casein fortified heavily with dl-methionine and with cysteine appeared at least equal to unfortified crude fibrin. A purified grade of blood fibrin (pharmaceutical) was slightly less effective in maintaining nitrogen balance than the crude grade.

[6] The author wishes to thank Drs. D. V. Frost and J. R. Schenck of this laboratory for supplying these dried hydrolysates.

[7] The author is indebted to Mr. E. O. Krueger for the tryptophane analyses.

TABLE 2

Summary of the nitrogen balance experiments.

A Nitrogen-containing supplement	B Protein or protein hydrolysate	C Total N [1] / Wt. of dl-methionine added	D Total N [1] / Wt. of cysteine $HCl \cdot H_2O$ added	E Dog no.	F Minimum intake which will produce N balance (in mg N/kg/day)
AA11	S.M.A. casein	No fortification		2	140–160
				17	
AA10	Beef blood fibrin (crude)	No fortification		3	70
				4	70
AA10'''	Fibrin, pharmaceutical grade	Not fortified		17	100
				18	100
				21	80
AA7	S.M.A. casein		4.90	5	100–120
AA8	S.M.A. casein		3.31	6	80–100
AA7a	S.M.A. casein	14.25	4.90	5	80
AA8a	S.M.A. casein	14.25	3.31	6	80
AA9	S.M.A. casein	7.11	4.90	2	70
				4	60
E-120c	Incompletely acid-hydrolyzed casein		3.31 [2]	4	80
E-120a	Incompletely acid-hydrolyzed casein		4.85 [2]	3	80
				4	80–100
E-201ds	Completely acid-hydrolyzed casein		4.85 [2]	3	80
				4	80–100
L-1542	Lactalbumin	Not fortified		10	100 [3]
				11	100 [3]

[1] Total N represents the nitrogen of the protein (in gm) before fortification. This is divided by the weight (in gm) of the amino acid used for fortification.

[2] l-Tryptophane was also added to the dried hydrolysate. The ratio

$$\frac{\text{Total N (in gm) of hydrolysate}}{\text{Wt. (in gm) of tryptophane present after fortification}}$$

was 11.0 for E-120c, 10.0 for E-120a, 12.0 for E-201ds.

[3] Lower levels of lactalbumin were not given and so this does not necessarily represent the minimum amount of lactalbumin nitrogen required to maintain nitrogen balance. These data were supplied by Dr. Frost of this laboratory.

TABLE 3

Nitrogen balance experiments.

DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED [1]	NITROGEN BALANCE	DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED [1]	NITROGEN BALANCE
	kg	*mg/kg/day*	*gm N/wk.*		*kg*	*mg/kg/day*	*gm N/wk.*
	Nitrogen containing supplement: AA11 — S.M.A. vitamin-free casein unfortified						
2	5.9	200	+ 1.88	17	9.2	120	— 3.92
	6.0	180	+ 1.21	(continued)	9.3	120	— 0.39
		160	+ 0.56		9.4	140	+ 1.11
	6.0	140	— 0.38		9.8	140	+ 1.25
	5.8	120	— 0.65		10.0	140	+ 0.95
	5.9	140	+ 0.13		10.3	160	+ 0.00
	6.0	160	+ 0.32		10.2	160	+ 2.11
	6.0	142	— 0.63		10.2	140	+ 0.87
	6.0	142	— 0.27		10.4	140	+ 1.43
	5.9	142	— 0.78		10.4	120	— 0.49
	5.9	159	— 1.13		10.4	120	— 0.17
	5.8	159	+ 0.49		10.5	100	— 0.67
17	9.3	140	— 0.13		10.4	100	— 2.03
	9.3	140	— 0.03		10.2	100	— 0.86
	9.0	120	— 0.47		9.9	160	+ 0.57
	Nitrogen containing supplement: AA10 — Beef blood, fibrin, crude						
4	8.5	100	+ 1.56	3	6.4	60	— 0.90
	8.5	80	— 0.25	(continued)	6.6	50	— 1.12
	8.4	70	+ 0.02	10	8.1	100	+ 1.08
	8.4	60	— 1.08		8.8	100	+ 0.41
3	6.4	125	+ 2.84		8.2	100	+ 0.74
	6.8	100	+ 1.28	11	7.7	100	+ 0.78
	6.6	80	+ 0.47		7.9	100	+ 0.35
	6.7	70	+ 0.07				
	Nitrogen containing supplement: AA7 — S.M.A. vitamin-free casein + approx. 2.9% of cysteine · HCl · H_2O						
5	6.1	80	— 1.67	5	6.3	100	— 0.57
	6.3	83	— 0.96	(continued)	6.3	120	+ 0.81
	6.5	80	— 1.32		6.3	120	— 0.07
	6.3	70	— 1.23		6.4	100	+ 0.30
	6.3	100	— 1.45		6.4	100	+ 0.60
	Nitrogen containing supplement: AA8 — S.M.A. vitamin-free casein + approx. 4.3% of cysteine · HCl · H_2O						
6	10.0	80	+ 0.21	6	10.3	76	— 0.70
	9.9	81	— 0.54	(continued)	10.2	100	+ 0.55
	10.3	70	— 2.31		10.2	100	+ 0.56
	10.2	60	— 1.72		10.3	100	+ 1.04
	10.2	80	— 1.15		10.5	80	— 0.37

TABLE 3 (continued)

DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED [1]	NITROGEN BALANCE	DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED [1]	NITROGEN BALANCE
	kg	*mg/kg/day*	*gm N/wk.*		*kg*	*mg/kg/day*	*gm N/wk.*
	Nitrogen containing supplement: AA7a — S.M.A. vitamin-free casein + approx. 1% of dl-methionine + 2.9% of cysteine · HCl · H_2O						
5	6.5	78	— 0.87	5	6.4	84	+ 0.41
	6.7	68	— 0.86	(continued)	6.4	83	+ 0.18
	6.3	70	— 0.73		6.5	80	+ 1.29
	6.4	70	— 1.37		6.4	84	+ 0.65
	6.5	80	— 0.29				
	Nitrogen containing supplement: AA8a — S.M.A. vitamin-free casein + approx. 1% of dl-methionine + 4.3% of cysteine · HCl · H_2O						
6	10.5	79	+ 0.06	6	10.5	86	+ 1.68
	10.5	69	— 0.73	(continued)	10.5	85	+ 0.99
	10.5	70	— 0.58		10.2	80	+ 2.46
	10.5	70	— 0.50		10.5	80	+ 1.87
	10.5	80	+ 0.35				
	Nitrogen containing supplement: AA9 — S.M.A. vitamin-free casein + approx. 2% of dl-methionine + 2.9% of cysteine · HCl · H_2O						
2	6.1	70	+ 0.66	2	6.3	70	— 0.50
	6.1	70	Urine sample lost	(continued)	6.3	70	+ 0.25
	6.1	60	— 0.55	4	8.4	81	+ 1.53
	6.1	50	— 0.63		8.4	70	+ 0.74
	6.4	70	+ 0.47 (9-day period)		8.5	60	+ 0.09
	Nitrogen containing supplement: E-120c — Dried partial acid hydrolysate of casein + approx. 4.3% cysteine · HCl · H_2O						
4	8.4	80	+ 0.50	4	8.4	50	— 0.51
	8.4	60	— 0.38	(continued)	8.0	50	— 0.58
	Nitrogen containing supplement: E-120a — Dried partial acid hydrolysate of casein + approx. 2.9% cysteine · HCl · H_2O						
3	7.2	100	+ 0.90	4	8.4	80	— 0.28
	7.2	80	+ 0.33		8.4	60	— 1.30
	7.1	60	— 0.65				

TABLE 3 *(continued)*

DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED [1]	NITROGEN BALANCE	DOG NO.	BODY-WEIGHT	AMOUNT OF NITROGEN FED [1]	NITROGEN BALANCE
	kg	*mg/kg/day*	*gm N/wk.*		*kg*	*mg/kg/day*	*gm N/wk.*
	Nitrogen containing supplement: E-201ds — Complete acid hydrolysate of casein + approx. 2.9% cysteine · HCl · H_2O						
3	6.9	100	+ 0.82 (6-day period)	4	7.6	80	— 0.64
	6.9	80	+ 0.07 (8-day period)		7.9	64	— 0.59
	6.8	60	Negative		8.2	48	— 1.81
	Nitrogen containing supplement: L-1512 — Lactalbumin						
10	8.4	100	+ 0.84	11	7.7	100	— 0.76
	8.7	100	+ 0.79		8.4	100	+ 0.42
	8.3	100	+ 1.17		7.7	100	+ 1.13
	Nitrogen containing supplement: AA10‴ — Beef blood fibrin, pharmaceutical grade						
17	9.0	79	— 3.21	18	7.5	80	— 0.24
	9.2	80	— 3.39	(continued)	7.5	80	— 0.09
	9.0	100	+ 0.23		7.5	100	+ 0.78
	9.0	80	— 2.14		7.5	100	+ 0.96
	9.0	80	— 0.89		7.5	100	+ 0.76
	8.8	80	— 0.71	21	9.5	79	— 0.09
	8.6	100	— 0.06		9.4	80	— 0.28
	8.5	100	+ 0.15		9.3	100	+ 1.53
	8.4	100	— 0.53		9.5	80	+ 0.58
18	7.6	61	— 2.18		9.4	80	+ 0.77
	7.6	79	— 0.94		9.4	80	+ 1.14
	7.6	80	— 0.62		9.4	100	+ 2.06
	7.6	100	+ 0.13		9.7	100	+ 2.71
	7.5	80	— 0.29		9.7	100	+ 2.33

[1] These figures were calculated from the "standard" or kennel weights of the dogs. The standard weights in kilograms of the dogs used are listed as follows: Dog 2, 6.9; Dog 3, 6.6; Dog 4, 8.4; Dog 5, 7.2; Dog 6, 10.0; Dog 10, 8.1; Dog 11, 7.5; Dog 17, 9.1; Dog 18, 7.2; Dog 21, 9.8.

In table 2 the results of the nitrogen balance experiments are summarized. In table 3 the results of the weekly nitrogen balance determinations are recorded. For each animal, these results appear in the order in which the experiments were carried out.

DISCUSSION

The literature on the protein minimum prior to 1937 has been adequately reviewed by Melnick and Cowgill ('37).

It is important to note that the animals we used for determining the protein minimum were in a protein depleted state, having gone through an adjustment period of 2 months to the low nitrogen intake of 100 mg of fibrin nitrogen per kg per day or 160 mg of casein nitrogen per kg per day. Their plasma protein levels were in the range of 5%. In the experiments of Melnick and Cowgill a 3- to 7-day period was used to allow the animals to adjust their metabolism to a new dietary regime. The level of nitrogen fed during the short adjustment period was never lower than 160 mg of casein nitrogen per kg per day or 150 mg of lactalbumin nitrogen per kg per day. The blood protein levels and general protein stores of the animals after this adjustment period were probably very nearly normal. Therefore, the protein minimums determined by Melnick and Cowgill were for dogs with normal protein storage supplies. The protein minimums we have determined are very close to the absolute minimums for survival when the animal receives adequate calories from carbohydrate and fat. If an animal is fed for prolonged periods of time less than the minimum levels of protein we have established, the animal's health deteriorates badly.

Although the protein minima for nitrogen equilibrium determined by the technique of Melnick and Cowgill are different from those we determined, their methods give results very similar to ours when one protein is compared to another. For example, taking the figures of Melnick and Cowgill and giving lactalbumin an arbitrary nutritional value of 100, the value found for casein was seventy-three. According to our findings, if 100 mg of nitrogen per kg per day is assumed to be the minimum amount of lactalbumin for nitrogen equilibrium (the minimum amount is probably very close to this), the relative nutritional value of casein is $\frac{100}{150} \times 100$ or 66.6.

From the cystine and methionine contents of casein and fibrin reported in the literature (Block and Bolling, '45),

(fibrin contains 1.5% cystine, 2.2% methionine; casein contains 0.3–0.5% cystine, 3.0–3.3% methionine) it is evident that the total content of sulfur amino acids in the two proteins is approximately the same. Yet, as described in the preceding paragraphs, we have found that casein is inferior to fibrin until it is fortified with cysteine and methionine whereupon it becomes equal to fibrin in ability to maintain nitrogen balance.

A possible explanation for this is that the cystine and methionine of casein are not well utilized, perhaps due to incomplete hydrolysis of the protein in the digestive tract. To test this point, the MPN value of a dried complete hydrolysate of casein fortified with only tryptophane should be determined and compared to that of unfortified casein. This has not been done, but a comparison of preparations E-120a and E-201ds (table 2), which are dried hydrolysates of casein fortified with tryptophane and cysteine, with AA7 (table 2), which is unhydrolyzed casein fortified with the same amount of cysteine, shows that the hydrolyzed preparations were slightly more effective in maintaining nitrogen balance. The experimental evidence on this point remains inconclusive however, because the hydrolysates were fortified with cysteine.[8]

It is claimed by Miller ('44) that cystine and methionine have a protein sparing action when added to diets very low in protein. Some such action may contribute to the greater effectiveness of casein fortified with cysteine and methionine in maintaining nitrogen balance.

Casein contains somewhat smaller quantities of threonine, tryptophane, phenylalanine, and leucine than does fibrin.

[8] After this paper was submitted for publication, Melnick, Oser, and Weiss (Science, vol. 103, p. 326, 1946) produced evidence that in vitro the enzymatic release of methionine from raw soy bean meal is slower than from heat-processed soy bean meal. The decreased nutritional value of raw soy bean meal would then be due to the late absorption of methionine. The same may be true of the sulfur amino acids of casein, thus providing another possible explanation for the beneficial effect of supplementing casein with methionine or cystine or both.

Since fortification of casein with adequate amounts of cysteine and methionine results in a mixture equally effective as fibrin in maintaining nitrogen balance, it is unlikely that casein is inferior to fibrin because it contains insufficient amounts of threonine, tryptophane, phenylalanine, or leucine.

It is not probable that strepogenin, the peptide-like growth factor for bacteria and mice, described by Sprince and Wooley ('44, '45, '45), played a role in these nitrogen balance studies. It is destroyed by acid hydrolysis of casein and it can be seen in table 2 that casein hydrolysates E-120a and E-201ds were slightly more effective in maintaining nitrogen balance than unhydrolyzed casein similarly fortified.

SUMMARY

By employing suitably prepared dogs, the minimum amount of a protein required to maintain nitrogen balance (MPN value) was determined.

The MPN values of casein, crude fibrin, and pharmaceutical grade fibrin were found to be, respectively, 140–160, 70 and 80–100 mg N/kg/day.

Fortification of casein with cysteine reduced the MPN value to 80–100 mg N/kg/day. When both cysteine and methionine were added to casein in sufficient amounts, the MPN value fell to 60–70 mg N/kg/day.

The MPN value for lactalbumin was not determined, but it maintained nitrogen balance when given at the rate of 100 mg of lactalbumin nitrogen/kg/day.

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INTRAVENOUS UTILIZATION OF PARTIAL ACID HYDROLYSATES OF PROTEINS[1]

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Investigations dealing with the efficacy of protein hydrolysates in intravenous nutrition have been reviewed by Elman ('44). Although no strictly quantitative data are available, there are indications that the peptide component of an enzymatic hydrolysate is utilized for anabolic processes. However, it is generally recognized that there is no definite proof of this Sahyun, '44).

White and Elman ('42) hydrolyzed casein and pumpkin seed globulin with sulfuric acid under mild conditions. Hydrolysis was incomplete but the major portion of the tryptophane remained intact under the conditions employed. When dried and fed to dogs, these hydrolysates maintained nitrogen balance. Recently, White and Sayers ('45) have reported further studies on incompletely acid-hydrolyzed proteins. These hydrolysates were injected rapidly into the veins of rats, guinea pigs, rabbits and dogs with no undesirable reactions resulting. No nitrogen balance studies were made however. Preparations of incompletely acid-hydrolyzed proteins will be called partial acid hydrolysates.

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By means of nitrogen balance studies in dogs, we have sought to determine whether the peptide component of a partial acid hydrolysate of a protein is utilized when the hydrolysate is administered by the intravenous route. This was done by determining the minimum amounts of complete and partial hydrolysates required to maintain nitrogen balance. A brief report covering part of this work has already been presented (Risser, Schenck and Frost, '46).

METHODS

The selection of animals, the preparation of animals for the nitrogen balance experiments, and the collection and analysis of urine and feces were done exactly as described previously (Risser, '46).

The complete hydrolysates were prepared by refluxing casein 4 days with 3.5 N sulfuric acid. The sulfate-free hydrolysate was shown to be substantially completely hydrolyzed, $\frac{\text{carboxyl nitrogen (ninhydrin)}}{\text{total nitrogen} - \text{ammonia nitrogen}} = 0.79$.

The partial acid hydrolysates were prepared by the method of White and Elman ('42) except that shorter refluxing periods were used (2 hours and 4 hours) in some experiments. The per cent of free amino acid nitrogen in these partial hydrolysates has been determined by Frost and Heinsen ('45).

Fortification of sulfate-free partial and complete hydrolysates with amino acids was based on a Kjeldahl nitrogen determination on the solution. The amino acids were added, the solutions were diluted to a volume convenient for use (5–7 mg N/ml) with pyrogen-free water, bottled, and quickly sterilized. The solutions were tested for pyrogens and another Kjeldahl nitrogen determination was obtained. The amounts of hydrolysate injected per day were based on this last Kjeldahl nitrogen determination (see seventh column of table 1).

The various types of fortifications with amino acids are given in table 1. This method of expressing the amounts of amino acids added to the hydrolysates may appear somewhat unusual, but it was used for the following reasons. The

amounts of amino acids were based on the nitrogen content rather than on the solid content of the hydrolysates because the percentage of total nitrogen varied slightly in different batches of the hydrolysates. The only accurate method of duplicating the amounts of amino acids added to new batches of fortified hydrolysate then was to base these additions on the nitrogen content. Likewise, since the amounts of the hydrolysates injected were expressed in milligrams of nitrogen per kilo per day, a better duplication of the amounts of fortifying amino acids administered per day was effected.

The amounts of amino acids used in fortifying can be approximately expressed in per cent of solids in the hydrolysate by assuming a total nitrogen of 14.5% for casein hydrolysates and a total nitrogen of 13.5% for fibrin hydrolysates. If Total N is divided by the ratios in the third and fourth columns of table 1, the result gives the percentage fortification. For example, hydrolysate E-214 contained $\frac{14.5}{12} = 1.2\%$ dl-tryptophane and was fortified with $\frac{14.5}{4.90} = 2.9\%$ cysteine monohydrochloride monohydrate.

The non-nitrogenous portion of the diet has been described (Risser, '46). A sufficient amount of this diet was given each day to provide 75 cal./kg/day. Daily records were kept of the amount of this diet consumed. The variation that occurred in the intake of this diet did not reflect in unusual weight changes (determined each week) in the animals and the amount consumed did not fall to levels which would be expected to affect the nitrogen balance results. The nitrogen contained in this portion of the diet amounted to less than 5% of the nitrogen provided by the intravenous hydrolysates.

For the intravenous injection of the hydrolysates each animal was fastened securely in an upright position to a frame designed especially for these experiments. The solution of the hydrolysate was poured into a sterilized burette (50-, 100-, or 250-ml capacity, depending upon the volume of solution to be given). The burette was equipped with a rubber tube, clamp and 20-gauge needle.

The needle was introduced into a leg vein. It was held in place with a strip of tape. The proper rate of flow of solution into the vein was obtained by regulating the clamp on the rubber tube and by adjusting the height of the burette. During the injection, the burette was periodically raised to keep the flow constant. The hydrolysates were given daily in one injection. This injection period in most of the experiments was 80 or 120 minutes (see table 1). With proper rotation of the use of the leg veins, it was possible to keep a dog continuously for 3 months on intravenous amino acids before the veins became difficult to use.

RESULTS

Solutions of completely hydrolyzed casein fortified with tryptophane and cysteine monohydrochloride monohydrate (solutions E-214 and E-215, table 1) were given intravenously to female dogs. The minimum intake of nitrogen which maintained nitrogen balance was found to be approximately 120 mg/kg/day.

Partial hydrolysates of casein (solutions E-185a and E-245 in table 1) were fortified with approximately the same amounts of cysteine as used in complete hydrolysates E-214 and E-215. Nitrogen balance was produced at an intake of 120 mg N/kg/day. Thus, although three-fourths of the amino acids present were bound in peptides (Frost and Heinsen, '45), they were available to the animal for the purpose of maintaining nitrogen balance.

Although hydrolysate E-185a was fortified with tryptophane to the same content of this amino acid in unhydrolyzed casein, this solution was no more effective than hydrolysate E-245 (table 1) which contained only the tryptophane left undestroyed by the hydrolysis. However, when the partial acid hydrolysate was not fortified with cysteine (solutions E-293 and E-298 in table 1), it was not as effective in maintaining nitrogen balance as the cysteine fortified hydrolysates.

A partial hydrolysate of fibrin was prepared using the conditions of White and Elman ('42). The amount of tryptophane

TABLE 1

Summary of nitrogen balance experiments.

HYDROLYSATE NO.	HYDROLYSATE	TOTAL N [1] WEIGHT OF TRYPTOPHANE PRESENT [2]	TOTAL N [1] WEIGHT OF CYSTEINE HCl·H_2O ADDED	DOG NO.	INJECTION PERIOD (MINUTES)	INTAKE OF HYDROLYSATE IN MG N/KG/DAY	BALANCE IN GM N/WEEK
E-214	casein, complete	12.0 (dl-tryptophane added)	4.90	1	80	100	— 0.94
					80	80	— 0.99
					80	120	— 0.49
E-215	casein, complete	12.0 (dl-tryptophane added)	3.31	2	80	100	— 1.33
					80	80	— 0.99
					80	120	+ 0.26
E-185a	casein, partial (6 hr.)	11.0 (dl-tryptophane added)	4.83	1	80	120	+ 0.33
					80	100	— 0.38
					80	80	— 0.62
				2	80	120	— 0.14
					80	140	+ 0.54
E-245	casein, partial (6 hr.)	25.0 (no tryptophane added)	4.83	3	80	140	+ 0.78
					80	140	+ 0.20
					100	120	+ 0.14
					90	100	— 0.05
				3	120	140	— 0.97
E-293	casein, partial (6 hr.)	23.8 (no tryptophane added)	no cysteine added		120	140	— 0.48
					120	140	— 1.17
				4	120	121	— 1.90
					120	120	— 1.39
					120	120	— 1.68
E-298	partial (6 hr.) of 1 part fibrin, 3 parts casein	18.4 (no tryptophane added)	no cysteine added	7	120	161	— 0.40
					120	140	— 0.34
					120	140	— 0.73
				8	120	148	— 0.51
					120	120	— 0.95
					120	120	— 1.69
E-270	crude fibrin, partial (6 hr.)	9.0 (no tryptophane added)	14.06	4	120	100	+ 1.02
					120	100	— 0.13
F-2B	fibrin, partial (2 hr.)	9.63 (no tryptophane added)	7.63	3	120	100	— 0.41
				7	120	120	+ 0.71
C-2B	casein, partial (2 hr.)	18.1 (no tryptophane added)	6.65	4	120	120	+ 0.61
					120	120	+ 0.95
F-4B	fibrin, partial (4 hr.)	7.42 (no tryptophane added)	5.75	4	120	120	+ 0.77
				8	120	130	+ 0.60
					120	100	+ 1.17
C-4B	casein, partial (4 hr.)	18.6 (no tryptophane added)	6.58	3	120	120	+ 0.45
					120	120	+ 0.34
				7	120	140	— 0.02
					120	140	+ 0.27
E-216	casein, complete	12.0 (dl-tryptophane added)	4.90 dl-methionine was also added. *Total N* = 14.2 dl-methionine added	4	120	120	+ 0.71
					120	100	+ 0.34
					120	100	+ 0.78
					120	80	— 0.16
E-201s	casein, complete	13.14 (dl-tryptophane added)	14.50 this ratio is for dl-methionine added. No cysteine added	1	80	120	— 0.15
				2	80	120	— 0.14
					80	100	— 1.44

[1] In this ratio, Total N represents nitrogen (in gm) of the hydrolysate before any additions of amino acids were made. Also, under Methods, see Fortification.

[2] The amounts of tryptophane in the partial hydrolysates were determined. "Tryptophane present" represents the amount (in gm) of the tryptophane retained in the partial hydrolysate plus the quantity added; for the complete hydrolysates, it represents the amount added.

left intact was much greater than in the casein hydrolysates similarly prepared.[3] The hydrolysate, fortified with cysteine (solution E-270, table 1), maintained nitrogen balance when given at the rate of 100 mg N/kg/day although over 60% of the amino acids were found in peptides (Frost and Heinsen, '45).

Both casein and crude fibrin were hydrolyzed by the method of White and Elman ('42) except that refluxing periods of 2 hours and 4 hours were used. Each hydrolysate was fortified with approximately the same amount of cysteine (see solutions F-2B, C-2B, F-4B, and C-4B, table 1). All of these hydrolysates were as effective as the 6-hour hydrolysates in maintaining nitrogen balance. The 2-hour fibrin and casein hydrolysates contained 20 and 17% free amino acids, respectively. Similar figures for the 4-hour fibrin and casein hydrolysates were, respectively, 33 and 20% (Frost and Heinsen, '45).

Complete casein hydrolysates E-216 and E-201s (table 1) represent different types of fortification with cysteine and methionine than were used in any of the partial hydrolysates studied. However, the results with these solutions further substantiate the fact that the minimum amount of a complete hydrolysate given intravenously capable of maintaining nitrogen balance is in the range of 100–120 mg N/kg/day.

Excretion of amino acids and peptides in the urine

We have not made a thorough study of the excretion of amino acids and peptides following the injection of these hydrolysates. However, in using the method of Van Slyke, MacFadyen and Hamilton ('43), we have found that after oral administration of fibrin or casein at the rate of 100 to 130 mg N/kg/day, 3 to 9% of the total nitrogen excreted was in the form of amino acid nitrogen. When the urine was hydrolyzed with 8 N H_2SO_4 for 18 hours, amino acid nitrogen was approxi-

[3] We wish to thank Mr. Elmer Krueger for all the tryptophane determinations appearing in this paper. They were done by the method of Shaw and McFarlane ('38).

mately doubled. Partial (6 hours) fibrin hydrolysates given intravenously at the rate of 120 mg N/kg/day in a 2-hour period gave approximately the same picture in amino acid and peptide excretion. When the hydrolysate was given in a much shorter period of time, there was a detectable increase in excretion of amino acids and peptides. For example, one dog received intravenously a partial hydrolysate of casein (6 hours) at the rate of 162 mg N/kg/day in 35 minutes. In the urine, 15.5% of the total nitrogen was found to be amino acid nitrogen. After hydrolysis with sulfuric acid, free amino acid nitrogen was found to be 28.7% of the total nitrogen.

Whether or not the extra amino acid nitrogen found after acid hydrolysis of urine actually originated from peptides we have not further investigated.

Reactions to these solutions, such as vomiting, were very infrequent, especially when the 2-hour injection period was used. Since the injections were made at a time when the animal's stomach was empty, the vomitus was in the form of mucus. This was mixed thoroughly with the urine for analysis. There was considerable variation in tendencies for different dogs to vomit.

DISCUSSION

Holman et al. ('34), Pommerenke et al. ('35), and Daft et al. ('38) have shown that a dog can be maintained in nitrogen balance when intravenously injected dog plasma is the only source of protein. Horse plasma protein was not similarly utilized. The mechanism of utilization of plasma protein for synthesis of other tissue protein was thought to involve cleavage of the plasma protein to large aggregates rather than to amino acids (Whipple, '38). It was postulated that these aggregates or peptides were used as building blocks to form specific cell protein or reserve store protein.

We believe that it is unlikely that the peptides of a partial acid hydrolysate are incorporated as such into tissue protein. It seems reasonable that they must be hydrolyzed to amino acids and that specific cell protein could be synthesized only from these amino acids. The literature shows that there are

polypeptides present in normal blood (Hiller and Van Slyke, '22; Godfried, '39; London and Kotschneff, '34) and polypeptidases (Grassmann and Heyde, '30; Abderhalden and Hanson,'37; Sennhenn, '41). Furthermore, it is reported that parenteral injection of peptides results in an increase of peptidase in plasma and serum (Hanson, '34). In the light of this information, it is not surprising to find that the peptides of partial acid hydrolysates are utilized by the dog after intravenous injection.

SUMMARY

We have determined the minimum amount required to maintain nitrogen balance of a complete acid hydrolysate of casein fortified with tryptophane and cysteine after intravenous injection into female dogs. The same was done for a partial acid hydrolysate of casein similarly fortified. Although 83 to 75% of the amino acids of the partial hydrolysate were bound in peptides, this partial hydrolysate was just as effective in maintaining nitrogen balance as was the complete hydrolysate given at the minimum level (120 mg N/kg/day), which would maintain nitrogen balance. This provides excellent evidence that the peptides of the partial acid hydrolysate are utilized for anabolic processes.

Partial and complete casein hydrolysates and partial fibrin hydrolysates were fortified in different ways with tryptophane, cysteine, and methionine and the effectiveness was studied of each fortified hydrolysate in maintaining nitrogen balance after intravenous administration.

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PROTEIN DEPRIVATION AS A CAUSE OF VASCULARIZATION OF THE CORNEA IN THE RAT

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TWO PLATES (TWELVE FIGURES)

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The various effects of protein deprivation or deficiency have been summarized by Metcoff, Favour and Stare ('45). We have recently reported (Sydenstricker, Hall, Hock and Pund, '46) that the appearance of corneal vessels in the rat may also be a result of protein deficiency, as well as of a deficiency of certain amino acids. While it is possible that corneal vascularization may result in man from inadequate protein nutrition, on biomicroscopic examination of some twenty malnourished individuals, all suffering from nutritional edema, none showed corneal vascularization. Lyle, McCrae and Gardiner ('44) found that the addition of about 230 gm of liver and 115 gm of kidney per week to the diet of eighty-two airmen for 10 weeks caused no significant improvement in the degree of corneal vascularization. Little corneal vascularity had been observed where the food was of good nutritive value but more was found where the food was less satisfactory.

Our present paper is an extension and amplification of our previous report on the occurrence of corneal vessels in rats suffering from a deficiency of protein (Sydenstricker, Hall, Hock and Pund, '46).

EXPERIMENTAL

Twenty-three rats of a Wistar strain were placed on a protein-free diet (diet 29, table 1) when from 50 to 62 days of age and thirteen rats from the same litters were fed the control diet (diet 31, table 1). We had previously observed that four rats placed on diet 29 when 30 days of age died before any very marked eye abnormalities could be seen with the biomicroscope (Sydenstricker, Hall, Hock and Pund, '46). Two out of three rats placed on the protein-free diet when 39 days of age had developed corneal vessels. The eyes of the rats

TABLE 1

Constitution of the protein-free diet (29) and control diet (31).

	DIET 29 PROTEIN FREE	DIET 31 CONTROL
Vitamin free casein (Labco)		240 gm
Sucrose	908 gm	668 gm
Salt mixture [1]	40 gm	40 gm
Cottonseed oil	30 gm	30 gm
Cod liver oil, U.S.P.	20 gm	20 gm
Choline chloride	2 gm	2 gm
Thiamine chloride	4 mg	4 mg
Pyridoxine hydrochloride	4 mg	4 mg
Riboflavin	16 mg	16 mg
Calcium pantothenate	20 mg	20 mg

[1] The salt mixture was as used by McKibbin, Madden, Black and Elvehjem ('39).

on the protein-free diet were examined every other day with the biomicroscope and the eyes of the group of control rats were examined weekly. During these examinations rats with typical eye changes were selected for injection or for studies of histological changes. The various techniques employed and the procedure in caring for the animals were as previously described (Bowles, Allen, Sydenstricker, Hock and Hall, '46).

In order to follow the regression of corneal vessels produced in rats fed the protein-free diet (diet 29), ten rats with varying degrees of vascularization were changed from the protein-

free diet to the control diet (diet 31). Resulting changes in the cornea were then followed with the biomicroscope and typical specimens were injected or taken for histological studies.

Three male rats 7 months of age were placed on the protein-free diet in order to determine the length of time necessary for corneal vessels to appear in the adult rat. Biomicroscopic examination of the eyes of these rats was made at weekly intervals.

In order to observe any relationship which might exist between the appearance of corneal vessels and the level of total plasma protein and blood hemoglobin, twenty rats were placed on the protein-free diet (diet 29) and seventeen rats from the same litters were placed on the control diet (diet 31). Blood samples for hemoglobin and protein determination were obtained by ventricular puncture from a sufficient number of rats in each group to provide a fair sampling of the group. The first sample was taken at the beginning of the experiment, a second, after corneal vascularization had appeared in most of the deficient rats, and a third after the deficient rats had been placed on the control diet and the vessels had regressed. Hemoglobin and total plasma proteins were determined by the methods of Evelyn ('36) and of Johnston and Gibson ('38), respectively. In the determination of total plasma proteins, the tyrosine factor for human blood was used, since Greenberg ('37) had found this factor for the rat and for man to be practically identical. The results of these determinations are summarized in table 2.

All the experimental animals were given food and water ad libitum and were kept in cages with wire bottoms, usually one or two animals to a cage. The rats were fed daily and weighed semiweekly.

In this investigation we have adhered to the practice which we earlier adopted (Bowles, Allen, Sydenstricker, Hock and Hall, '46) of limiting our use of the term "corneal vascularization" to the situation where the extent of corneal capillary invasion exceeds the limits of normal variation. These limits

TABLE 2

Average total plasma protein and blood hemoglobin levels in rats on deficient and control diets. All values are in gm per 100 ml.

	CONTROL RATS DIET 31		DEFICIENT RATS DIET 29	
	Hemoglobin	Plasma protein	Hemoglobin	Plasma protein
At beginning of experiment	13.28 ± 0.54 (13)[1]	5.67 ± 0.20 (13)	13.23 ± 0.39 (19)	5.47 ± 0.18 (19)
At time of appearance of vascularization	14.91 ± 0.53 (10)	5.86 ± 0.11 (11)	12.34 ± 0.57 (10)	4.93 ± 0.19 (9)
After vascularization had regressed through feeding diet 31 to deficient rats	14.80 ± 0.29 (9)	6.14 ± 0.19 (8)	12.98 ± 0.73 (7)	6.08 ± 0.25 (7)

[1] Numbers in parentheses indicate the number of rats on which the determinations were made.

we determined by examining the eyes of some 500 normal rats.

RESULTS AND DISCUSSION

The ocular changes observed in rats on the protein-free diet were not entirely uniform and seemed to depend to some extent on the size and age of different litters of rats and, at times, to differences among litter-mates.

In the majority, the first definite change was edema of the scleral conjunctiva; often this was marked; sometimes it was not observed. Usually a day or two later there was congestion of the conjunctiva with engorgement of the circumferential vessels of the limbic area. Often there was a slight and usually transient thickening and opacity of the cornea. This may have been due to edema alone or to edema plus leukocytic infiltration. In a few animals corneal opacity was striking and nebulae were seen in addition to diffuse opacity. Whether or not corneal opacity was observed, in about a week,

sometimes as soon as 2 days after the development of conjunctival congestion, there was evidence of beginning invasion of the cornea by new-formed capillaries.

Frequently these capillaries stemmed from the circumferential vessels opposite the points where feeder vessels were visible; almost as often "sprouts" from the circumferential artery appeared at numerous points widely separated from the "feeders." There was no uniformity in the process of corneal vascularization. In the majority of rats a dozen or more capillaries sprouted from the circumferential artery and rapidly invaded the superficial area of the cornea. These capillaries seemed to lie immediately beneath the corneal epithelium and, as they grew larger, often produced ridges on the surface of the cornea. Eventually, they reached the center of the cornea and anastomosed with capillaries invading from the opposite side. In a few instances the vessels assumed a dendritic appearance with clusters of capillary loops terminating the invading vessel. In these rats a rather dense collar of vessels eventually was formed extending about halfway from the limbus to the center of the cornea. The peak of corneal vascularization seemed to be reached some 2 to 3 weeks after the first evidence of ocular involvement. After this, in rats continued on the protein-deficient diet, there was a period of 2 to 3 weeks during which corneal opacity recurred, sometimes as diffuse cloudiness, sometimes as localized nebulae.

Treatment with the control diet (diet 31) produced rapid regression of corneal vascularization. The new-formed vessels in the cornea grew narrower, there was "beading" of the blood columns in them, and eventually the vessels became invisible or were represented by fine white streaks in the cornea, probably the endothelial columns persisting after patent capillaries ceased to be visible. Concurrently such opacity as existed cleared up, leaving a perfectly transparent cornea. As might have been expected, narrowing of the invading capillaries and eventual disappearance was much more rapid when treatment was begun before the ultimate grade of vas-

cularization was reached. In some animals a few large anastomosing vessels have persisted for weeks after all the network of finer capillaries had become invisible.

Figures 1 to 8 are anterior and oblique views of injected corneas from four rats, which show different degrees of the development of corneal vascularization due to protein deprivation. It was noted in corneal preparations from rats suffering from protein deprivation that the newer portion of the injected capillary near the center of the cornea appeared larger in diameter than the rest of the capillary. This was in contrast to what was observed in vitamin A and riboflavin deficiencies where the newer portion of the injected capillary tended to be smaller. Since in all cases the injection pressure was approximately the same, this may mean that the newer portion of the capillary is more readily distensible, or on the other hand it may be merely an indication of the increasing severity of the deficiency during the development of the capillary. Once invasion of the cornea begins, the time required for the capillaries to penetrate nearly to the center of the cornea is roughly the same in vitamin A, riboflavin or protein deficiencies. However, the period of previous deficiency before invasion begins is long in vitamin A and riboflavin deficiencies, while in protein deprivation this period is rather short and almost equals the time required for extensive invasion. The degree of deficiency of the animal is probably increasing much more rapidly during the corneal capillary invasion in protein deprivation than with the vitamin A and riboflavin deficient rats.

The suggestions which we made in an earlier paper (Bowles, Allen, Sydenstricker, Hock and Hall, '46) as to the pattern of vascularity in riboflavin and vitamin A deficiencies depending on the progress of the capillary invasion of the cornea and the development of capillary branches, seem to apply also in the vascularization resulting from protein deprivation. The general pattern of the vessels as observed in each of these three deficiencies appears to be very similar, though there is some difference in detail. We also have observed in these prep-

arations the tendency of the capillaries to arch over the points where the feeder vessels bifurcate to form the circumferential vessels as we found in vitamin A and riboflavin deficiencies. In figures 5 and 7 it may be observed that vascularization has proceeded more rapidly in two quadrants than in the other two. This also is in accord with our observations with other deficiencies. Showing faintly in the photographs used for figures 7 and 8 were small vessels anastomosing over the center of the cornea. Since the injection fluid did not enter these vessels, it may be that they were not yet functional.

Figures 9, 10, 11 and 12 show three stages in the regression of corneal vessels which resulted when the deficient rats were changed to the control diet. At the time the injection preparation shown in figures 9 and 10 was made, the vessels had diminished in size and were bloodless, but the injection pressure was sufficient to force the ink into the vessels as far as they had been observed to extend on biomicroscopic examination. The cornea shown in figure 11 developed vessels extending two-thirds of the way to the center while on the deficient diet. At the time of injection these were also much diminished in size and bloodless. As may be seen, the vessels were opened to half of their length by the injection fluid. Figure 12 shows a cornea which had been vascularized nearly to the center and was similar in degree to the vascularization shown in the cornea in figures 7 and 8. At the time of injection the vessels were bloodless and were no longer visible with the biomicroscope. It may be seen, however, that the injection fluid penetrated a small portion of the proximal end of some of the capillaries which were much diminished in size. A further part of some of the capillaries showed faintly in the photograph even though not filled with injection fluid.

A discussion of the changes in histology of the cornea resulting from protein deprivation will be included in a later paper.

In the rats which were 50 to 62 days of age, corneal vessels appeared in all the rats on the protein-free diet in from 9 to 20 days. Corneal vessels developed in the three adult rats

at 24 days, 38 days and 59 days, respectively. Four rats which had been placed on the protein-free diet when 30 days of age died without developing corneal vessels (Sydenstricker, Hall, Hock and Pund, '46). It appears that the age of the rat when placed on the diet and the degree of deficiency are important factors in determining whether or not corneal vascularization results, as well as the length of time required for corneal vascularization to develop, just as with all other nutritional deficiencies so far studied (Bowles, Allen, Sydenstricker, Hock and Hall, '46).

In the course of the biomicroscopic examinations of the rats' eyes it became evident that the animals suffered from a very definite photophobia which appeared approximately at the time when significant eye changes were first observed. We (Bowles, Allen, Sydenstricker, Hock and Hall, '46) had observed a similar photophobia in rats suffering from vitamin A or riboflavin deficiency as had other investigators.

None of the thirty rats on the control diet (diet 31) showed more than normal variation in the width of the limbic area and in none of these was corneal vascularization ever observed. Since that time, we have had thirty other rats on this control diet, some for as long as 4 months, of which only one developed a mild degree of corneal vascularization.

Rats on the control diet (diet 31) grew an average of 2.2 gm per day as compared with 2.4 gm per day for a group of nine rats of the same strain and age which were on a diet of a commercial dog chow,[1] lettuce, carrots and milk. The rats on the protein-deficient diet (diet 29) lost an average of 1.3 gm of weight per day.

It was observed that many of the rats on the control diet (diet 31) developed a rusty, disheveled appearance and, in some cases, shed part of their hair. These rats were individually fed a daily supplement supplying 1 mg per day each of inositol, para-aminobenzoic acid and nicotinic acid. Within 10 days the rats had resumed a normal appearance. One mg daily of riboflavin, or of nicotinic acid alone had no such ef-

[1] Purina.

fect. Control rats in other experiments with similar diets, where the carbohydrate was supplied as starch rather than the sucrose used here, never developed the disheveled appearance described above. The significance of these observations is not entirely clear, though the explanation probably involves intestinal synthesis of substances important to the nutrition of the rat by the intestinal flora. Mannering, Orsini, and Elvehjem ('44) found that with rats on a diet suboptimal with respect to riboflavin, sucrose minimized growth and fecal excretion of riboflavin as compared with starch or dextrin as a source of carbohydrate, indicating that the starch favored intestinal synthesis of the vitamin while sucrose minimized this effect. Similarly, McIntire, Henderson, Schweigert and Elvehjem ('43) ascertained that the growth of rats on a synthetic diet containing limiting amounts of thiamine was stimulated by the administration of para-aminobenzoic acid and inositol, presumably due to increased intestinal synthesis of thiamine. It thus seems logical to assume that some substance needed by the rat was produced by the intestinal flora when starch was the dietary carbohydrate in our diets or when p-aminobenzoic acid and inositol were administered, but not with sucrose in the absence of p-aminobenzoic acid and inositol.

The data in table 1 show that at the time of appearance of corneal vascularization, the rats on the protein-free diet had undergone a significant reduction in blood hemoglobin and total plasma protein levels. However, the drop of 0.89 gm in hemoglobin level and of 0.54 gm in total plasma protein level is probably not of great practical importance as such. The work of Metcoff, Favour and Stare ('45), and of Chow, Allison, Cole and Seeley ('45) would indicate that the decreased levels of hemoglobin and plasma protein were accompanied by a considerable decrease in the amount of circulating blood. The decrease in the plasma protein level probably is a result of a decrease in the albumin fraction only (Chow, Allison, Cole and Seeley, '45; Zeldis, Alling, McCoord and Kulka, '45). By the time when the corneal vessels had

regressed, the total plasma protein had returned to a level not significantly different from that of the control rats. The hemoglobin level had risen but was still below that of the control rats.

The rise in hemoglobin level in the control rats during the course of the experiments is in accord with what one might expect in rats of this age, since according to Creskoff, Fitz-Hugh and Farris ('42) newborn rats average 75% hemoglobin (Sahli); then, after birth, there is a slow irregular increase toward the adult level of 101% (100% = 15.6 gm per 100 ml of blood).

SUMMARY

Rats 50 days of age or older, when placed on a diet devoid of protein, develop extreme corneal vascularization. By the time the vascularization appears, blood hemoglobin and total plasma protein show a slight decrease in level. When the rats are returned to an adequate diet, the vessels regress.

ACKNOWLEDGMENT

This study was aided by grants from the John and Mary R. Markle Foundation and from Merck and Co. We wish to thank Dr. Harris Isbell, Dr. Sam Singal, Dr. Philip Dow and Dr. Lane Allen for suggestions and assistance. Elizabeth J. Thompson cared for the animals used and Nell C. Murphey gave technical assistance.

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PLATE 1

EXPLANATION OF FIGURES

1 to 6 Anterior and oblique views of injected corneas from three rats, showing vascularization resulting from protein deprivation. The rats had been on the protein-free diet for 20, 20, and 37 days, respectively. (× 15 reduced approximately a third).

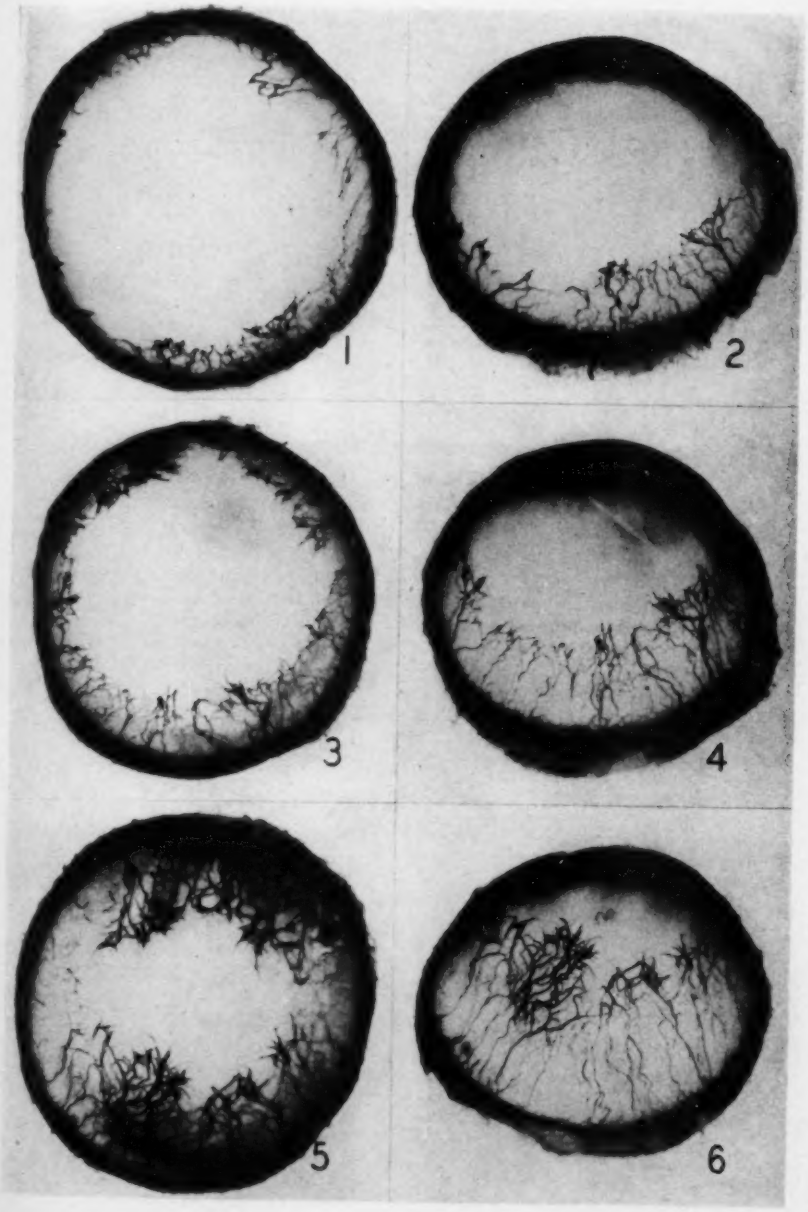
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PLATE 2

EXPLANATION OF FIGURES

7 and 8 Anterior and oblique views of an injected cornea from a rat showing vascularization resulting from protein deprivation. The rat had been on the protein-free diet for 37 days.

9 Anterior view of injected cornea shown in figure 10.

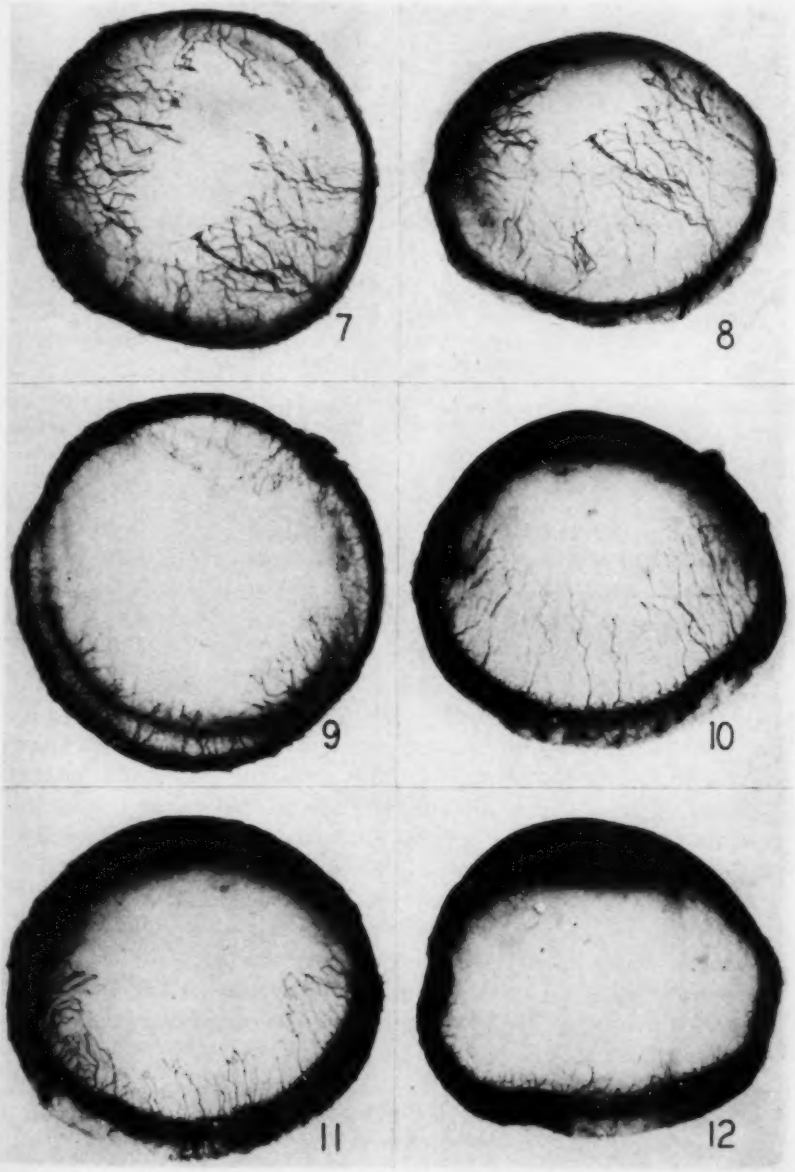
10 to 12 Oblique views of injected corneas from three rats showing regression of vascularization. The cornea in figures 9 and 10 is from a rat which had been on the protein-free diet 36 days followed by 22 days on the control diet.

The corresponding periods for the others rats were:

11 Protein-free diet, 32 days and control diet, 13 days.

12 Protein-free diet, 36 days and control diet, 115 days.

(All × 15 reduced approximately a third.)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DENTAL CARIES IN THE SYRIAN HAMSTER

II. A PRELIMINARY STUDY OF THE EFFECT OF THREE DIFFERENT RATIONS ON CARIES ACTIVITY [1]

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TWO FIGURES

(Received for publication July 5, 1946)

Carious lesions in the molar teeth of Syrian hamsters apparently are associated with many of the fundamental caries-producing mechanisms common to human lesions (Keyes, '46). In previous work a low incidence of caries in hamsters has been associated with appreciable amounts of fluoride in either food or drinking water (Dale, Lazansky and Keyes, '44; Dale and Keyes, '45). In their natural environment hamsters are omnivorous but live largely upon whole grains which they gather in fields and store in burrows. Part of the present study was designed to determine the caries-producing effect of a whole grain diet of low fluoride content. Sugar was incorporated in a known caries-producing ration to determine whether any appreciable difference in caries activity followed its addition to the diet.

Dry yellow corn was selected as a low-fluoride whole grain ration. The Hoppert-Webber-Canniff formula ('32), previously found to be caries-producing was used for purposes of comparison. Sucrose was added to the latter mixture to

[1] This work was made possible, in part, by a grant of the Eastman Dental Dispensary of Rochester, N. Y. Abstract presented before the Twenty-third General Meeting of the International Association for Dental Research, Chicago, Ill., May 27, 1945.

determine whether its incorporation in the diet affected caries activity.

EXPERIMENTAL

All hamsters were obtained from an inbred colony maintained on a stock diet of commercial rabbit food.[2] From twenty litters between 4 and 5 weeks old, 100 animals were distributed at random into five groups of nineteen to twenty-one animals each. In the three groups used for this study, females ranged from 36 to 72 gm in weight (av. 49 gm); males, from 35–58 gm in weight (av. 48 gm).

Group I animals (12 females and 8 males) were given a basal ration of whole yellow corn. After the sixth day one-half of the group (6 females and 4 males) received supplements of a 90% whole powdered milk, 10% dry powdered alfalfa mixture. From the sixth to thirteenth day this supplement was available ad libitum. The allowance was then restricted to 20 gm per animal per week. The fluoride content of the milk-alfalfa mixture was 1.64 ppm.

Group II animals (12 females and 7 males) received the standard Hoppert-Webber-Canniff (H. W. C.) diet: 60% corn meal; 30% whole powdered milk; 6% linseed meal, 3% alfalfa meal, and 1% NaCl. A screened sample of the corn meal was distributed as follows: 100% through a 20-mesh screen; 40% on a 40-mesh screen; 10% on a 60-mesh screen; and 50% through a 60-mesh screen.

Group III animals (12 females and 8 males) were fed a diet containing two parts of the H. W. C. mixture and one part of confectionery sugar.

Rations were analyzed for moisture, protein, fat, ash, and fluoride by official methods of the A. O. A. C. These values are reported in table 1 together with estimations calculated from reliable sources (Sherman, '41; Jacobs, '44; Handbook of Nutrition, '43; and The Canned Food Reference Manual, '43).

Animals were kept in metal cages with wood shavings for bedding. Diets and distilled water were available ad libitum.

[2] Purina Rabbit Checkers.

TABLE 1

Percentage values of some constituents in diets.

RATION	HOPPERT-WEBER-CANNIFF	HOPPERT-WEBER-CANNIFF AND SUGAR	WHOLE CORN	NIBBLED CORN	EDIBLE CORN FRACTION
Moisture	7.6	5.2	10.6	11.1	9.9
Protein	17.4	11.6	10.1	9.4	11.5
Fat	10.8	7.2	4.0	1.4	9.2
Carbohydrate and N.F.E.[1]	56.	71.	73.4	73.9	53.2
Ash	4.6	3.1	1.3	0.5	2.9
Fluoride	0.87 ppm	0.92 ppm	0.61 ppm 0.25 ppm [2]	0.16 ppm [2]	0.43 ppm [2]
Calcium [1]	.313–.321	.208–.214	.015–.029	.016	
Phosphorus [1]	.408–.498	.272–.332	.28 –.43	.007	
Vitamin A [1]	10 I.U./gm	6.6 I.U/gm	2–8 I.U./gm	7–7.5 I.U./gm	
Vitamin D [1]			0	0	0
Thiamine [1]	4.2 μg/gm	2.8 μg/gm	5.4 μg/gm	.5–3 μg/gm	
Niacin [1]	12 μg/gm	8 μg/gm	2–26 μg/gm	+	
Riboflavin [1]	5.3 μg/gm	3.5 μg/gm	1.4 μg/gm	+	

Fresh alfalfa supplements supplied additional amounts of Ca and P, Vitamins A, E, K, etc.

[1] Values calculated from Sherman, '41; Jacobs, '44; Handbook of Nutrition, '43; and The Canned Food Reference Manual, '43.

[2] Comparable values in a corn sample obtained after completion of this experiment.

Supplements of fresh green alfalfa were supplied to each group once a week. The animals were maintained on these rations for 100 days after which time they were sacrificed.[3] From heads fixed in 10% formalin, the jaws were removed, stripped of soft tissue, and dried. The teeth were examined under a low-power dissecting microscope (1.5 × 10), and all gross carious lesions were charted and scored by a method previously reported (Keyes, '44).

RESULTS

With one exception all animals survived the experimental period. A severe epidemic of "sniffles" passed through the

[3] One female in Group I died on the fifty-fifth day of the experiment. There was no evidence of caries in the teeth at this time. These data are not included in final results.

entire colony during the sixth and seventh week and retarded growth slightly. Otherwise the animals remained in good health.

All Group I hamsters on whole corn grew poorly, except those which received the milk-alfalfa supplement. Weight curves of hamsters on the H. W. C. formula (Group II) were the best of the three groups. Animals fed the H. W. C. ration mixed with sugar (Group III) grew better than those on whole corn but not so well as those on the standard H. W. C. ration (fig. 1).

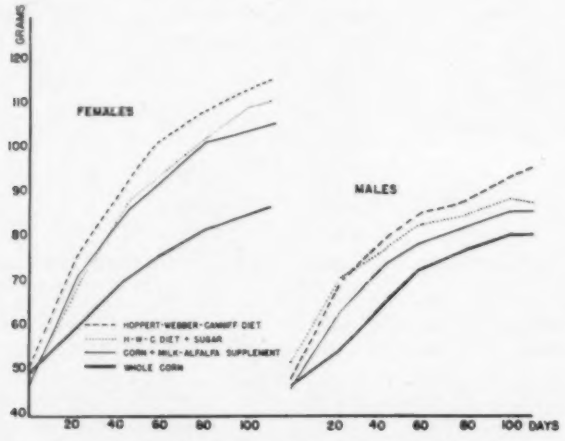

Fig. 1 Growth curves for male and female hamsters.

The incidence of dental caries in the three groups is indicated in tables 2 and 3. The whole corn ration was not conducive to caries activity. Since the milk-alfalfa supplement did not affect the incidence of tooth decay favorably or unfavorably, all caries values for Group I are reported together. The H. W. C. ration, prepared with a semi-fine corn meal, was not high caries producing. A marked increase in caries activity was found in animals that received sugar in their ration. Not only were a greater number of teeth involved, but the number of cavities and extent of cavitation were also increased.

TABLE 2

Caries incidence as found in individual molars.

MOLAR TEETH	GROUP I		GROUP II		GROUP III	
	% molars affected	Ave. % of teeth decayed	% molars affected	Ave. % of teeth decayed	% molars affected	Ave. % of teeth decayed
			Female			
Maxillary						
1st	0	0	0	0	62.5	6.1
2nd	0	0	4.3	0.09	75.0	34.8
3rd	0	0	0	0	83.5	45.4
Mandibular						
1st	0	0	0	0	62.5	6.4
2nd	8.4	0.2	33.3	0.4	79.1	17.2
3rd	0	0	0	0	54.1	7.7
			Male			
Maxillary						
1st	0	0	0	0	56.2	5.2
2nd	0	0	21.4	2.8	62.5	31.1
3rd	6.2	0.7	71.5	10.0	87.5	59.5
Mandibular						
1st	0	0	7.2	3.6	62.5	20.1
2nd	50.0	3.1	93.0	25.1	68.8	10.7
3rd	0	0	35.7	1.2	68.8	21.7

TABLE 3

Caries incidence in relation to experimental diets — Summary table.

	GROUP I Whole corn		GROUP II H. W. C. diet		GROUP III H. W. C.-sugar	
Number of animals in group	11♀♀	8♂♂	12♀♀	7♂♂	12♀♀	8♂♂
Percentage of animals affected	18	62	58	100	100	100
Percentage of carious teeth	1.5	9.3	6.3	38.1	69.4	67.7
Average number of carious lesions	0.2	1.3	0.7	4.6	10.3	11.1
Average score [1]	0.1	1.8	0.2	19.3	51.1	64.1

[1] Of a possible score of 282, representing complete molar crown destruction.

Under the conditions of this experiment, male hamsters showed a tendency to be more susceptible to dental caries than female. This difference was most apparent in Groups I and II. In Group III the difference is less apparent and is suggested only when the over-all average of scores is considered.

DISCUSSION

As a preliminary approach to more detailed studies, this experiment was designed to compare the effect of three different diets on the incidence of dental caries in hamsters. The chemical constituents reported in table 1 are but a partial indication of the total nutrients in the rations. However, from these values alone it would be difficult to predict the effect of these diets on caries activity. There appears to be no definite correlation between chemical constituents and the caries experience of the different groups. The type and activity of the fluoride compounds in the rations is not known, but apparently they were not present in sufficient quantity to exert a caries-inhibiting effect.

Maxillary second and third molars and mandibular second molars showed a high degree of susceptibility, but sufficient data are not available to determine the relative susceptibility of each tooth. No explanation of the sex difference in caries activity can be offered at present. Arnold ('42) found more lesions in male than in female animals but did not conclude that a sex difference existed. Dale and Keyes ('45) also report such findings.

Hamsters eating the dried corn nibbled out the germ and consumed only small amounts of the hard endosperm. The slow growth of animals on this ration suggested nutritional deficiencies.[4] Despite the possible inadequacies of this ration, animals on this diet had as little evidence of caries activity as we have observed. To conclude that the low incidence of dental caries in this group is due to an optimum or even adequate diet is difficult. It is evident, however, that neither

[4] There has been complete growth failure in a small group of hamsters subsequently maintained on a whole corn ration without supplementation of any kind.

the physical nature of the cereal particles comminuted by the animals nor the chemical composition of the kernel fraction consumed was conducive to the processes that initiate carious lesions.

Weight curves are used as acceptable criteria of animal progress on a given regimen. In dietary studies poor growth generally indicates the lack of sufficient food or the absence of essential nutrients. Since rations were available ad libitum, the poor growth in Group I was undoubtedly due to the scarcity of essential nutritional constituents. The suggestion is sometimes advanced that a high incidence of caries is the result of dietary inadequacy and lack of protective foods. On this basis one would expect to find high caries scores in animals which grew the poorest and low scores in those which grew the best. The opposite was found in this study. The possibility has been suggested that the greater incidence of caries in males may indicate that the rations did not contain the most desirable concentration of nutrients needed by the hamster, and therefore, male animals, which presumably grow more and have a greater demand for essential nutrients, were more susceptible to tooth decay. If this were the case, one would expect to find the highest incidence of caries in males on whole corn and the lowest incidence of caries in males whose weight curves exceeded females, e.g., males on the H. W. C. and H. W. C.-sugar rations compared with females on whole corn. However, male animals with poor growth had very little evidence of caries and those whose growth exceeded the corn-fed females had 200 to 600 times more tooth substance destroyed. This presumption is untenable, however, because male hamsters normally weigh less than the females.

That inadequate diets may not be associated with dental caries has been reported for human beings. A low incidence of dental caries has been observed in people suffering from severe rickets, osteomalacia, and other nutrition deficiencies (Taylor and Day, '39; Day, '44; Staz, '38).

Although the H. W. C. ration has been adequate for rats (Hoppert, Webber and Canniff, '32), the complete adequacy of this diet for hamsters has not been tested. Animals receiving this ration have remained in good health and shown no symptoms of nutritional deficiency (Dale, Lazansky and Keyes, '44; Dale and Keyes, '45). Since typical rickets can be produced in hamsters only on low phosphorus and vitamin D deficient rations (Jones, '45), this diet cannot be considered rachitogenic, especially when supplemented with fresh greens. In contradistinction to rats, however, caries activity is not associated with cusp fractures from coarse corn meal in the diet and actually increases on finer corn meal. In part, this variation may be due to differences in crown morphology and type of occlusion between the two species.

In this experiment the caries experience of animals on the H. W. C. ration was considerably lower than expected from previous work. A factor influencing the caries-producing properties of this diet may have been the semi-fine grind of the corn.[5] The particle size of one-half the corn meal was too large to collect in the sulci and fossae of the teeth (fig. 2). Such particles may have had a detergent action.

The effect of sugar on experimental caries in rats has been confusing. After extensive survey of the literature on rat caries, Cox ('44) concluded that sugar will promote the enlargement of cavities but fails to cause the initial appearance. However, Shaw and coworkers ('44) and Schweigert et al. ('45) report a high incidence of caries activity in cotton rats fed high percentages of sugars in diets. Recently, McClure ('45) reported that white rats developed a significant incidence of microscopic caries when fed synthetic diets containing excessive quantities of sucrose and glucose.

In hamsters the addition of powdered sugar greatly increased caries activity and affected not only the incidence of

[5] The corn meal used in previous studies (Dale et al., '44; '45) sifted as follows: 24% on a 40-mesh screen, 13% on a 60-mesh screen, and 63% through a 60-mesh screen.

lesions but also the size. Whether the sugar per se was responsible for this increase in caries activity or whether its combination with other constituents in the ration formed a caries-producing combination is not known. The data indicate that the mixture of sucrose into the diet was conducive to initiation of lesions as well as development.

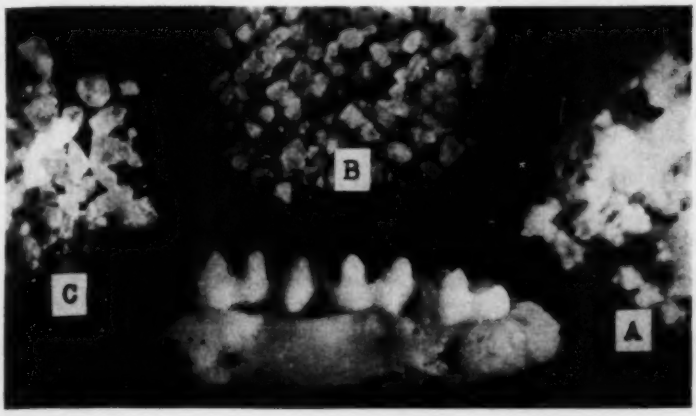

Fig. 2 Three maxillary molars in situ compared with corn meal fractions in the Hoppert-Webber-Canniff diet: A. corn fraction passing 60-mesh screen, 50%; B. corn fraction retained by 60-mesh screen, 10%; C. corn particles retained by 40-mesh screen, 40%.

CONCLUSIONS

Data from this experiment suggest that, in hamsters, caries activity is related more to the dietary constituents than to the nutritional value of ingredients. The physical character of the ration and the sex of the animal apparently influence caries activity. Emphasis is placed on the observation that there may be no correlation between growth curves and caries activity. Inadequate rations are not necessarily caries-producing, and rations which support satisfactory growth may have high caries-producing value.

ACKNOWLEDGMENT

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THE GROWTH AND MAINTENANCE UTILIZATION OF DIETARY PROTEIN [1]

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FIVE FIGURES

(Received for publication March 13, 1946)

The biological value of protein has been defined as the fraction of the absorbed protein nitrogen that is retained in the body (Thomas, '09). This expression of the nutritive quality of proteins as it has been modified by Mitchell ('24) may be applied to either growing or adult animals. However, in the adult the retained nitrogen is utilized only for the maintenance of the nitrogenous integrity of the tissues while in the growing animal increases in body protein as well as maintenance requirements account for the retained nitrogen. The purpose of this investigation was to measure the maintenance and growth utilization of several proteins in young rats and to study the influence of the amount of protein ingested.

EXPERIMENTAL

Weanling male albino rats [3] were individually caged in a room with temperature controlled at 78°F. and were fed a commercial diet [4] for 3 days. This pre-test period was included so as to provide time for some readjustment of the

[1] Parts of this paper were reported at the American Chemical Society meeting, New York, 1944, and the New York Academy of Sciences, New York, 1945.

[2] Present address: Sharp and Dohme, Inc., Glenolden, Pennsylvania.

[3] Sprague-Dawley.

[4] Purina Fox Chow.

TABLE 1

Composition of the basal diet.

Protein sources that were incorporated in the diet replaced carbohydrate and fat so that the respective combinations were isocalorie in nature.

BASAL MIXTURE		VITAMIN MIXTURE [1] PER 100 GM OF BASAL MIXTURE	
			mg
Sucrose: varied inversely with protein added		Riboflavin	400
Fat [2]	15%	Thiamine	400
		Pyridoxine HCl	400
		Niacin	2,500
Salt mixture [3]	4%	Calcium pantothenate	1,100
Fiber [4]	2%	Para amino benzoic acid	7,500
Vitamins A and D [5]	+	Choline HCl	200,000
Vitamin mixture [1]	+	Inositol	20,000
		2-Methyl 1,4 naphthoquinone	50
		alpha Tocopherol	2,500

[1] The tocopherol was added to the fat and the water-soluble components were incorporated in the sucrose.

[2] Soybean oil was added to make a total of 15%.

[3] Wesson ('32) modification of the Osborne-Mendel salt mixture with a trace of $ZnSO_4$ added.

[4] Cellu flour, from Chicago Dietetic Supply Co.

[5] Cod liver oil was administered by mouth with an eyedropper twice weekly.

animals to their new environment. The rats were then divided according to weight into twenty-eight groups of eight rats each so that the average body weight of each group was approximately 60 gm. Four crude protein sources [5] representing a wide range in nutritive quality were incorporated into the basal diet described in table 1, so that seven different

[5] Spray-dried whole eggs extracted three times with cold petroleum ether. The protein content (N × 6.25) was 76%.

Soyflour (no. 1) kindly supplied by Dr. J. Hayward, Archer-Daniels-Midland Co., Minneapolis, Minn. The analysis was protein (N × 6.25) 48.9% and fat 0.3%. The product was commercially processed so as to develop a high nutritive quality protein.

Soyflour (no. 2) kindly supplied by Dr. J. Hayward, Archer-Daniels-Midland Co., Minneapolis, Minn. The analysis was protein (N × 6.25) 49.3% and fat 0.3%. The product was commercially processed so as to develop a protein of intermediate nutritive quality.

Wheat gluten purchased from the Huron Milling Co., Harbor Beach, Michigan. The analysis was protein (N × 6.25) 75%.

levels of each protein could be fed. Each diet was checked for protein content by Kjeldahl nitrogen determinations. For 42 days the rats ate these diets ad libitum and careful observations of body weight and food consumption were made. Feces were collected over 4-day periods during the second, fourth, and sixth weeks and apparent digestibility was calculated from the difference between the total intake and fecal output of nitrogen. An average of the three fecal collection periods was used to determine the total protein apparently absorbed during the 42-day period.

At the conclusion of the test period the rats were killed with ether and after washing out the contents of the stomach and large intestine the carcasses were digested with sulfuric acid and Kjeldahl nitrogen was determined. Carcass nitrogen was also measured on a group of rats at the start of the experiment so that calculation of gains in carcass protein during the 42-day experimental period could be made.

RESULTS

Determination of body protein gain

With increased amounts of the four crude protein sources in the diet there was an increase in protein consumption (see table 2). The increased consumption of protein enhanced the growth rate until a maximum was obtained. The maximum body protein gain was essentially the same for three of the crude proteins, but the poorly heated soyflour (soyflour no. 2) supported a maximal protein gain that was distinctly below the others (see fig. 1). The anomalous result may be related to the peculiar influence of heat upon the nutritive quality of soybean protein. With increasing consumption of dried egg protein it would appear that body protein gain reached a maximum and then decreased slightly. Decreased growth and body protein gain at high levels of protein intake have been observed in other studies (MacKay et al., '41; Bosshardt et al., '46).

TABLE 2

Growth and maintenance data derived from studies on 4 crude protein sources. Values are expressed as averages per rat for 42 days.[1]

1	2	3	4	5	6	7	8
GROUP NO.	PROTEIN IN DIET	AVERAGE BODY SURFACE AREA	PROTEIN CONSUMED	PROTEIN APPARENTLY ABSORBED	BODY PROTEIN GAINED	PROTEIN FOR MAINTENANCE (EGG)	PROTEIN FOR ENERGY ALONE
	%	cm^2	*gm*	*gm*	*gm*	*gm*	*gm*
			Whole egg				
1	4.1	182	7.4	6.3	1.5	5.3	—0.5
2	5.8	220	16.9	15.0	9.1	6.4	—0.5
3	7.9	247	25.9	23.6	16.0	7.1	0.5
4	9.9	286	39.8	35.8	27.2	8.3	0.3
5	13.7	297	57.6	51.8	29.7	8.6	13.5
6	19.0	297	77.4	70.5	30.7	8.6	31.2
7	38.7	303	153.0	142.0	29.0	8.8	104.0
			Soyflour no. 1				
1	6.0	188	14.7	12.5	3.9	5.5	3.1
2	7.7	204	23.8	20.2	6.5	5.9	7.8
3	9.4	227	31.8	27.3	11.7	6.6	9.0
4	11.5	242	41.0	35.7	15.1	7.0	13.6
5	14.8	262	59.5	51.7	20.5	7.6	23.6
6	18.5	280	79.6	69.3	25.6	8.1	35.6
7	27.7	286	117.0	100.0	27.9	8.3	63.8
			Soyflour no. 2				
1	6.1	164	12.4	9.7	3.4	4.7	1.6
2	8.5	172	19.3	15.8	5.5	5.0	5.3
3	9.7	185	24.0	19.7	7.5	5.3	6.9
4	12.6	207	37.5	31.6	12.6	6.0	13.0
5	14.5	234	55.6	46.3	18.0	6.8	21.5
6	16.9	232	58.8	49.4	19.5	6.7	23.2
7	31.1	230	98.0	82.3	19.3	6.6	56.4
			Wheat gluten				
1	8.4	173	20.2	19.2	1.1	5.0	13.1
2	10.2	178	21.6	20.5	2.0	5.1	13.4
3	12.4	181	27.1	25.8	2.6	5.2	18.0
4	14.6	188	33.4	31.7	3.8	5.4	22.5
5	20.8	211	56.8	54.0	7.4	6.1	40.5
6	31.5	242	106.0	102.0	15.2	7.0	80.0
7	43.0	287	187.0	179.0	27.5	8.4	143.0

[1] Column 1 — Eight rats in each group.

Column 2 — Protein (N × 6.25) determined by Kjeldahl analysis of each diet.

Column 3 — Average body surface area during the 42-day experimental period. Each figure is also the average of the group of eight rats.

Column 4 — Ad libitum food intake corrected for spilled food and protein intake calculated from analysis of the diets.

Column 5 — Protein apparently absorbed calculated according to the equation: protein intake minus fecal protein = protein apparently absorbed.

Column 6 — Body protein gained determined from carcass nitrogen at the end of the experiment minus carcass nitrogen of control group taken at the start of the experiment.

Column 7 — This calculation is based on the observation that maintenance requirements for protein are proportional to body surface area. Furthermore, it is assumed that whole egg protein at low levels in the diet is entirely used for maintenance. Therefore, at any given body size the amount of protein used for maintenance alone is equal to the mg of egg protein (N × 6.25) that was found necessary for the maintenance of nitrogen equilibrium (average of two values given in table 3) multiplied by the body surface area in square decimeters. The entire calculation of protein used for maintenance during the experimental period is: (mg egg N per 100 sq. cm. B.S. per day) × (protein conversion factor) × (days of experimental period) × (average body surface area during experimental period in square decimeters). An example of this calculation using group no. 1 receiving soyflour no. 1 (table 2) is: 11.1 × 6.25 × 42 × 1.88 = 5500 mg protein.

Column 8 — It is assumed that protein neither used in building new tissue nor maintaining the integrity of tissues is utilized directly as a source of energy. The calculation in this column represents the difference between column 5 and the sum of columns 6 and 7.

Calculation of the maintenance utilization of protein

The second major use of dietary protein is in the maintenance of the nitrogenous integrity of the tissues. This type of protein utilization may be defined as the minimum amount of absorbed protein that will sustain exact nitrogen equilibrium; in other words maintain the animal without overall gain or loss of body protein. The body protein gains that are described in figure 1 may be used to calculate the minimum

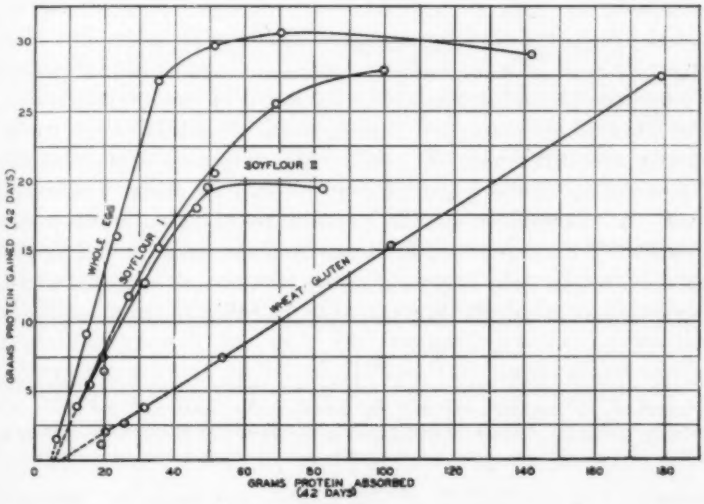

Fig. 1 Gain in body protein following the ad libitum ingestion of rations containing varying amounts of protein.

maintenance requirements of young, 60-gm rats, by extrapolating the curves to the point of zero body protein gain. This is in essence the method used by Osborne and Mendel ('15) for the measurement of the maintenance requirement. It has been shown by others (Olson and Palmer, '40; Smuts, '35) that the so-called endogenous urinary nitrogen excretion varies directly with body size. Furthermore, the best correlation appears to be with body surface area. It was considered

possible that the maintenance requirements established by extrapolation may have been influenced by reduced caloric intake due to the lowered protein content of some of the diets. Also it was possible that these maintenance requirements would not remain a constant function of surface area over the wide range of body size from immature to adult rats. Therefore, four groups of adult rats of the same sex and strain as used in the above study were fed diets containing the four crude protein sources at low levels that would either maintain the rats in approximate nitrogen equilibrium or would produce a slightly negative nitrogen balance. By plotting nitrogen balance against nitrogen apparently absorbed it was possible to interpolate to the point of exact equilibrium. The general principle of this method of establishing maintenance requirements of adult rats was the same as that described by Melnick and Cowgill ('37) in their studies on dogs. A comparison of the protein requirements for maintenance of nitrogen equilibrium as measured by extrapolation of protein gain in young rats and the conventional nitrogen balance in adults is presented in table 3 (first section).

TABLE 3

Comparison of protein requirements for the maintenance of nitrogen equilibrium as measured by two different methods involving the adult and the young rat (columns 2 and 3). Columns 4 and 5 give a comparison of the nutritive values of four protein sources for maintenance of nitrogen equilibrium and for growth.[1]

PROTEIN SOURCE	NITROGEN BALANCE (ADULT)	CARCASS NITROGEN (YOUNG)	MAINTENANCE (ADULT)	GROWTH (YOUNG)
1	2	3	4	5
	mg N/100 sq. cm [2]			
Whole egg	11.2	11.0	100	100
Soyflour no. 1	13.6	13.2	83	55
Soyflour no. 2	15.1	15.3	73	45
Wheat gluten	18.0	17.6	62	21

[1] Relative maintenance values are calculated from data on the minimum nitrogen intake necessary for the maintenance of nitrogen equilibrium (columns 2 and 3) with whole egg protein arbitrarily set at 100. Relative growth values are calculated from the maximal protein efficiency ratios (Barnes et al., '45) with whole egg protein arbitrarily set at 100.

[2] Body surface area estimated from the formula Surface Area = 11.36 × Body Weight $^{2}/_{3}$.

When expressed as a function of surface area the results are essentially the same for both methods. The curves of body protein gains for the two soyflours that are presented in figure 1 show that the extrapolation comes to exactly the same point for both protein sources. The rats receiving the soyflour no. 2 diets were started at body weights that were approximately 10 gm lighter than the others. When expressed as a function of surface area there was a definite difference in the maintenance requirements for the two proteins.

Graphic representation of protein utilization

The results of the above study substantiate the conclusions of others that maintenance requirements are directly proportional to body surface area. Furthermore they provide a basis for the calculations of the amount of protein that was utilized for maintenance of the nitrogenous integrity of the tissues during the 42-day growth period that was used in this study. The average body surface area of each group of rats during the 42-day experimental period was calculated from the growth curves.[6] The assumption was made that whole egg protein is 100% utilized for maintenance at low levels of intake and the average amount of protein utilized for maintenance of each of the experimental groups was established on this basis. An illustration of this calculation is presented in a footnote to table 2. The assumption that whole egg protein is utilized as a perfect protein for maintenance may be open to criticism but the work of Mitchell and Carman ('26), Murlin et al. ('38) and others indicates that the utilization of this protein mixture approximates 100%.

With this assumption it was possible to express the maintenance and the growth utilization of protein as a percentage of the protein apparently absorbed. Also the difference between the sum of these two types of utilization and the total absorbed protein must represent protein that was directly

[6] Growth curves for the rats used in these studies have been presented elsewhere (Barnes et al., '45).

utilized for energy. The data that are pertinent to these calculations are given in table 2 and the graphic representation of the three types of utilization for the four crude protein sources is given in figures 2, 3, 4 and 5. The expression "waste energy" has been used to designate the protein that was not used for growth or maintenance. The term "waste" is intended only to indicate a type of utilization that is not unique to proteins.

According to concepts developed by Mitchell the biological value of a protein is the sum of the nitrogen utilized for maintenance and growth expressed as a percentage of the absorbed nitrogen, the latter being corrected for metabolic fecal nitrogen. Absorption data presented here have not been corrected for metabolic fecal nitrogen but it is probable that the qualitative relationships that will be dealt with are not affected by this omission. This value which is the complement of "waste energy" also has been represented graphically in figures 2, 3, 4 and 5. In each case the curves have been extrapolated to the theoretical level of protein absorbed where there would be zero body protein gained. In the case of egg protein the maintenance at this point was assumed to account for 100% of the absorbed protein. In the other examples the percentage of absorbed protein utilized for maintenance at this level of protein intake was calculated from the maintenance data that were presented earlier. In each case the biological value is entirely accounted for by the maintenance value. According to Allison and Anderson ('45) the biological value should remain at this same level through the range of negative nitrogen balance to the point of zero protein intake.

At levels of protein intake in excess of the amount required for maintenance alone there is a sharp increase in the percentage utilization for body protein gain until a maximum is reached. This maximum is clearly defined for all proteins except wheat gluten. In this case body protein gain appears to establish a plateau. Percentage utilization for maintenance

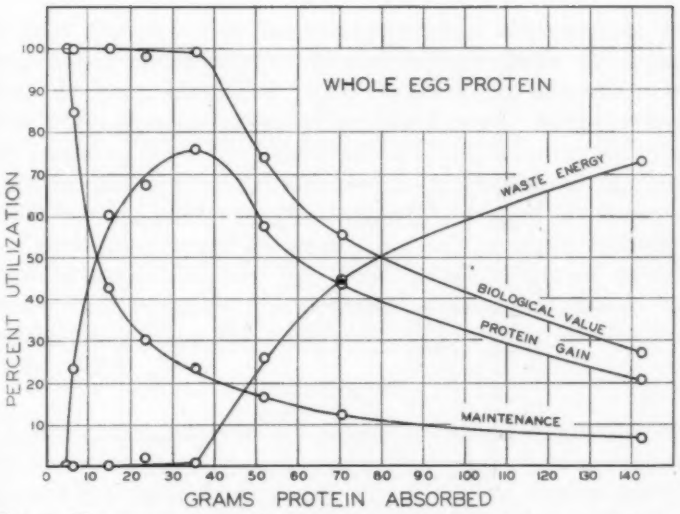

Fig. 2 The comparative utilization of whole egg protein for maintenance and growth.

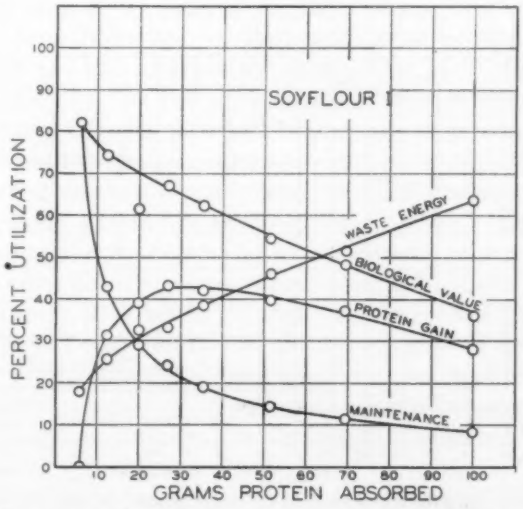

Fig. 3 The comparative utilization of a well heat-treated soyflour protein for maintenance and growth.

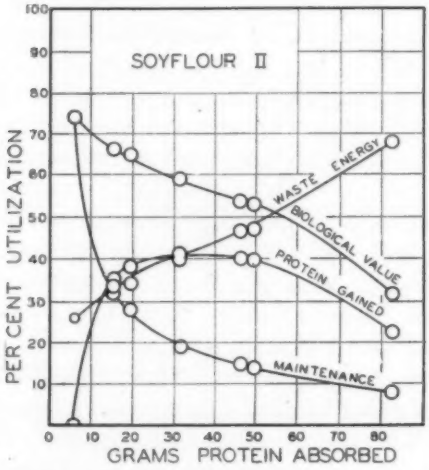

Fig. 4 The comparative utilization of a poorly heat-treated soyflour protein for maintenance and growth.

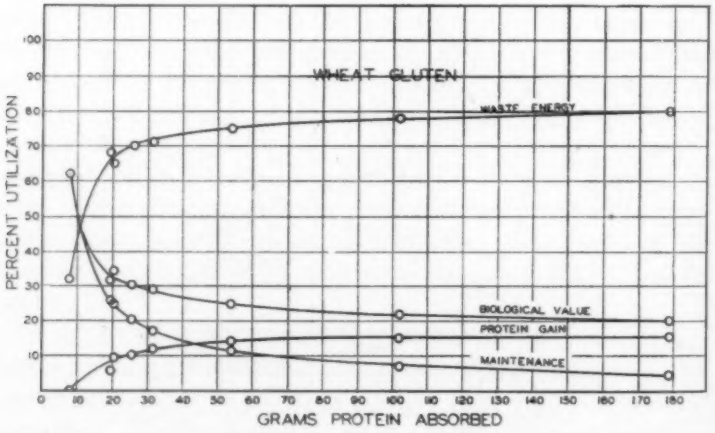

Fig. 5 The comparative utilization of wheat gluten protein for maintenance and growth.

declines sharply with increasing protein consumption and approaches an asymptote at the higher levels of intake. The resultant biological value exhibits an immediate decline when protein intake is increased except in the case of egg protein. Here the biological value remains in the proximity of 100 until maximal growth utilization is attained. Following this comes the characteristic decline. The observation that this protein source has a fairly constant biological value of approximately 100 over a wide range of protein intake lends support to the original assumption that it was completely utilized for maintenance at a very low level of intake.

DISCUSSION

The observation that the biological value of proteins decreases when the level of intake is increased substantiates Mitchell's ('24a) conclusions. The graphical representation of changes in biological value and in component utilizations that make up the biological value offers a more thorough understanding of the reasons for the change.

There is a difference in the quantitative protein requirements for growth and maintenance. This is a well known observation and the results presented here provide further confirmation. If the nutritive value of whole egg protein for both growth and maintenance is arbitrarily set at 100 and the relative values for the three remaining protein sources are calculated, the relationships given in table 3 (second section) are obtained. It will be seen that the peak utilization of the poorest protein source, wheat gluten, for maintenance is approximately 62% while the maximal utilization for growth is only 21%. These data are too incomplete to be used in drawing detailed conclusions regarding the relationship between requirements for growth and maintenance, but they do show the marked difference in the amounts required for these two types of utilization.

If the biological value of a protein is measured in growing animals and is, therefore, composed of a growth as well as maintenance utilization, the relative participation of these

two factors will vary, depending upon the amount and the quality of the protein that is fed. The changes in growth and maintenance utilization with increasing consumption of protein is readily seen in figures 2, 3, 4 and 5. Keeping in mind the differences in protein requirements for growth and maintenance this demonstrates that the significance of the biological value as well as the actual numerical value that is obtained is dependent upon the level of protein-intake. The change in the relative participation of growth and maintenance to the biological value with different quality proteins is shown in table 4. With egg protein approximately 77% of the biological value is due to growth and 23% to maintenance. With

TABLE 4

Relative participation of growth and maintenance in the establishment of the biological value when approximately 10% protein is included in the diet.

PROTEIN SOURCE	PROTEIN APPARENTLY ABSORBED PER RAT/42 DAYS	BIOLOGICAL VALUE	RELATIVE PARTICIPATION OF GROWTH	RELATIVE PARTICIPATION OF MAINTENANCE
	gm	%	%	%
Whole egg	36	99	77	23
Soyflour no. 1	27	67	65	35
Soyflour no. 2	20	64	59	41
Wheat gluten	20	35	26	74

[1] Calculated from data in either column 6 or 7 of table 2 at the level of each protein in the diet that is nearest to 10%.

the lower quality proteins this ratio decreases so that with wheat gluten only 26% of the biological value is due to growth while 74% is due to maintenance. These data are for rats that are receiving a diet composed of approximately 10% protein. Equalization of the protein intake may reduce these differences slightly, but will not abolish them. These results indicate that the significance of the biological value is not the same for proteins of different nutritive qualities. The conclusion that is drawn from these studies is that the measurement of the combined nutritive quality of dietary proteins for growth and maintenance may result in a numerical evaluation having an uncertain significance. The interpretation of values

would be more definite if growth and maintenance utilization of proteins were determined separately.

In the above calculations of growth and maintenance utilization of dietary protein it has been recognized that these two metabolic processes cannot be clearly separated. However, the existence of the two types of protein utilization has been clearly demonstrated and it would appear that there is sufficient evidence to warrant the conclusion that these two metabolic processes operate simultaneously in the growing animal.

The absolute values for the maintenance utilization of dietary protein that are presented here are derived by calculation and include the assumption that at low levels of intake, egg protein is completely utilized for maintenance. Protein utilization is influenced by a great variety of conditions and part of the values presented here have been derived by indirect calculation. Therefore, stress has been put on the qualitative relationships that were observed rather than implying that the absolute maintenance utilization and the biological values were calculated with extreme accuracy. In spite of the indirect approach that was used it is interesting that the biological values for the four test proteins compare favorably with values that are to be found in the literature.

SUMMARY

1. A study has been made of the growth and maintenance utilization of four crude protein sources covering a wide range of nutritive qualities.

2. When the level of protein intake is increased there is a decline in the fraction of absorbed protein that is utilized for maintenance. At the same time the fraction utilized for growth rises to a maximum and then declines. The net result of these changes is a fall in the biological value.

3. The relative participation of growth and maintenance in making up the biological value varies markedly depending upon the quantity and the nutritive quality of protein that is ingested.

4. Since the protein requirements for growth and maintenance are different the conclusion is drawn that these two factors should be measured independently and not in combination.

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OBSERVATIONS ON PELLAGRA IN AMERICAN PRISONERS OF WAR IN THE PHILIPPINES

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The surrender of Bataan and Corregidor in April and May of 1942 brought approximately 17,000 Americans under the control of Japanese Forces. During the subsequent 3 years of confinement in Japanese prison camps in the Philippines deficiency diseases developed in great numbers.

For 3 months prior to capture, the American Forces were on a diet restricted to about one-half the usual field ration. It consisted principally of polished rice plus small amounts of flour, sugar, and canned foods. Caloric values often fell to less than 1000 cal. per day and there was a marked deficiency in fresh meat, fruits and vegetables.

Malaria was rampant among the troops, and cases of bacillary and amebic dysentery appeared in increasing numbers. The fever and digestive disturbances associated with these diseases contributed to deficiency states.

Toward the end of the 5-month campaign symptoms such as night blindness and nutritional edema became common and occasional cases of mild peroneal paralysis with foot drop appeared. But symptoms of pellagra were not seen.

After the surrender, the Americans received from the Japanese during the summer and fall of 1942 a ration consisting

[1] The authors were serving as medical officers with U.S. troops on Bataan and were captured and held by the Japanese from April 9, 1942 to January 30, 1945. The observations included in this paper were made during that time.

essentially of the following: polished rise, 300 gm; vegetables (greens, leaves and vines), 100 gm; flour, 30 gm; sugar, 10 gm; and oil, 10 gm. Approximately once a week an average of 10 gm of fresh meat per man was issued. Thus the diet afforded less than 1500 cal. and it was hopelessly deficient in proteins and vitamins.

Nutritional edema became universal in the first few weeks of prison camp existence varying in degree from mild pedal edema to generalized anasarca. Beri-beri appeared by July, 1942, and it remained always the most severe and widespread deficiency entity.

Symptoms of pellagra first appeared with dramatic suddenness in August, 1942. Within a week 500 men out of a camp of 5000 presented signs and symptoms of the disease. They complained chiefly of soreness and burning of their mouths and tongues aggravated by hot or seasoned foods.

The tip of the tongue presented the typical raw, spotted appearance due to swollen, inflamed papillae. The inflammatory lesion progressed, extending along the margins of the tongue, along the median sulcus and to the under surface. Later ulcerations appeared on the tongue. The angles of the mouth became macerated and presented a perleche-like membrane. A seborrheic lesion appeared about the alae of the nose and spread over the lips and chin. The posterior aspect of the ears was similarly involved in some cases.

The skin lesions seen were similar to but more extensive than those usually described as pellagrous. They occurred on parts of the body exposed to sunlight and they were aggravated by sunlight. An erythematous dermatitis appeared on the dorsum of the hands, on the forehead, and in a butterfly pattern over the cheeks and nose. A similar lesion developed on the prominences of the back, chest and abdomen, on the dorsal aspect of the arms and feet, on the anterior thighs, and on the calves of the legs when these areas were exposed. Protection from sunlight by clothing or the straps of wooden clogs or sandals left a pattern of relatively uninvolved skin sharply demarcated from the pellagrous lesion.

In the more severe cases the mucous membrane of the entire mouth and pharynx became inflamed and edematous. There was a blotchy, reddish-purple, granular appearance to the palate and pharynx. Patches of grayish pseudomembrane were often present. The tongue was swollen, raw and fiery red having a beefy appearance. It was often crenated from pressure against the teeth. Speaking, chewing and swallowing were painful and difficult.

In the areas of dermatitis the subcutaneous tissue became soggy with edema. Vesicles and bullae appeared. Often the bullae became hemorrhagic. Desquamation, rupture of the bullae, weeping eczematous lesions, and secondary infection with crusting, purulent lesions marked the progression of the disease. Other areas of the skin, principally the flexor folds of the knees and elbows, assumed a moderately edematous, blotchy, ecchymotic appearance. We were unable to demonstrate a significant increase in capillary fragility by the tourniquet test.

Skin integrity was so impaired that the slightest trauma would result in a break in the integument with infection and ulcer formation. We believed that nicotinic acid deficiency was an important factor in the etiology of the so called "tropical ulcers" which were seen in great numbers among the prisoners.

Dermatitis of the scrotum was an interesting condition observed as a prodromal as well as a concomitant symptom of pellagra. Men came to sick call complaining of a burning, itching scrotum. Examination revealed an inflamed, edematous, weeping dermatitis of the scrotum which would dry and desquamate or become secondarily infected and exude a sanguino-purulent fluid. The usual signs of pellagra might not appear for as long as 3 weeks later.

The fact that the scrotal dermatitis was alleviated, at least in part, by protection of the scrotum from the friction of clothing again demonstrated the decreased resistance of the skin to trauma. A similar dermatitis occurred in a perianal distribution.

In cases less severe or improving the erythema was followed by thickening, roughening and scaling of the skin which frequently cracked and became infected. There was an associated pigmentation similar to sun tan. During remissions the skin often had a glossy, atrophic appearance.

Many patients exhibiting mild clinical signs of pellagra or giving a previous history of pellagra suffered from digestive disturbances. They complained of postprandial epigastric discomfort, flatulence, pyrosis, foul eructations described as "yeasty" or like rotten eggs, and recurrent attacks of diarrhea. In the presence of so many cases of infectious diarrhea and dysentery it was difficult to decide in individual cases of pellagra whether the diarrhea was on an infectious basis or whether it was a symptom of the pellagra. It was believed that the deficiency resulted in inflammation and irritability of the gastrointestinal tract with impaired digestive efficiency and diarrhea and with an increased susceptibility to infection. Diarrhea, whether on a functional or infectious basis, resulted in aggravation of deficiency symptoms.

Later when scant laboratory facilities became available, it was demonstrated that many pellagrins had a reduction or absence of free hydrochloric acid. The impression was that this impairment of gastric secretion was part of the pellagra syndrome. Dilute hydrochloric acid, when available, definitely alleviated these symptoms. The decrease in gastric acidity was possibly a factor in the high incidence of intestinal infestation encountered.

Cases of idiopathic urinary frequency and dysuria with occasional hematuria were common and may have been due to a bladder lesion associated with a generalized involvement of all mucous membranes.

Neurological lesions were common in pellagrous patients, but to what extent these were attributable to thiamine deficiency it is difficult to say. Sensory changes consisted of numbness and tingling, decreased sensation to light and deep touch, and loss of position sense and sensory ataxia. Trophic changes often persisted after subsidence of the dermatitis.

Some showed paresis, foot drop and impairment of deep reflexes while others showed spasticity and paresis and exaggerated deep reflexes. These changes occurred for the most part in the lower extremities.

Symptoms of irritability, moodiness, loss of memory and inability to concentrate which were observed might be ascribed either to deficiency or to psychic trauma from privation, abuse, and crowded confinement. A few patients progressed to disorientation and delirium.

These patients were suffering from a mixed deficiency and it is difficult to estimate how much of the neurological lesions was attributable to nicotinic acid deficiency and how much to thiamine deficiency. Symptoms of peripheral nerve lesions responded to thiamine therapy and symptoms of central nervous system lesions responded to nicotinic acid therapy. In addition, it was our impression that there was considerable overlapping of effects.

Pellagrins exhibited decreased resistance and often succumbed to malaria and dysentery, but no deaths were thought to be due to pellagra primarily.

Although the clinical signs of the disease were slow in manifesting themselves (4 to 8 months) the course of the disease once established was relatively rapid. At Cabanatuan, the symptoms appeared about mid August and by mid to late September some men were exhibiting the more serious lesions. Eventually, the greater part of the command suffered from symptoms to some degree.

Individual susceptibility to dietary deficiency was marked. Men of Spanish and Italian extraction, which races characteristically live on a high carbohydrate diet, were less affected, possibly because of some innate or hereditary quality. Too, the individuals of lighter complexion were more susceptible to the pellagrous lesions, particularly those of the skin. As groups, the larger men and the men doing hard physical work were more susceptible to vitamin deficiency. The men seemed to make a metabolic adjustment to the type of diet and fared better in later months. Actually this may have been due to

the survival only of those physiologically and psychologically capable of living on the diet.

Again it must be emphasized that practically all prisoners exhibited some evidence of B complex deficiency. It was more severe in those who suffered the ravages of dysentery and recurrent malaria and in those who were unable to get even small quantities of food from outside sources. Anyone who could not supplement the Japanese ration of rice and greens soon fell prey to severe pellagra.

The Japanese medical officer showed curiosity and interest in our problem but our request for relief in the form of a more adequate diet as well as specific medication met with little sympathy.

We were given a few bottles of Japanese compressed yeast tablets and we were able to obtain a few bottles of brewer's yeast from Filipino sources. Response to this medication was quite rapid even in amounts as little as 2.0 to 4.0 gm per day. Unfortunately, our supply was utterly inadequate to cope with the great number of cases which developed. Consequently, a yeast culture was prepared using a thin liquid medium composed of cornstarch, sugar, rice and water, seeded with brewer's yeast and wild yeast and allowed to grow for 3 to 5 days.

The number of patients far exceeded our supply of yeast culture, which was limited by lack of materials for the vats as well as for the medium. We were able to treat only the severe cases and those only until a remission with alleviation of the more advanced lesions was obtained. The dosage of the yeast culture varied from 100 to 250 ml depending upon the number of patients being treated. At all times we attempted to obtain and set aside a small amount of extra food for the more serious cases. Surprising improvement followed daily administration of the yeast culture alone or with the addition to the diet of a banana, a handful of peanuts, or part of a cocoanut when these were available. Most cases showed immediate retardation of the disease process with some improvement in 2 to 3 days and marked improvement in 7 to 14

days. Protection from sunlight was an important factor in the healing of the dermatitis.

In December, 1942, American Red Cross supplies of food and medicine arrived in the prison camp. We received enough canned and preserved food to allow an auxiliary daily ration for a period of 2 months of approximately 30 gm of canned meat, 100 gm of canned vegetables, and 20 gm of dried fruit. At about this time the Japanese began almost daily issues of 25 to 50 gm of fresh carabao meat per man and they increased the rice issue to about 500 gm per day. The ration now amounted to about 2000 cal. with 35 gm of protein of which about 10 gm was meat protein. During these months the pellagrins improved rapidly and markedly. The symptoms completely disappeared in most of the patients only to reappear in a milder chronic form in the lean days that followed. In this disease as in the other deficiency diseases we experienced, the value of processed foods for relieving deficiency states was far greater than we had anticipated.

Nicotinic acid was available in limited amount for the first time. In the stubborn cases in whom symptoms persisted even after the improvement in diet, 25 to 100 mg was usually sufficient to obtain relief of symtoms. However, a few were only partially or temporarily controlled on as much as 500 mg per day. The latter were usually of a blonde or sandy complexion.

Nicotinic acid proved to be an effective aid in the treatment of oral sepsis particularly trench mouth. The cheilosis, the para-alar seborrheic dermatitis and the dermatitis of the malar eminences usually attributed to riboflavin deficiency were found to respond to nicotinic acid as well as to yeast therapy. This again brings out the difficulty of classifying lesions according to specific vitamin lack and the suggestion of a high degree of overlapping effect of the vitamins of the B complex.

Those patients who were shown to have decreased or absent free hydrochloric acid by gastric analysis were treated with dilute hydrochloric acid orally and nicotinic acid, thiamine and liver extract parenterally. Laboratory studies in-

dicated improved gastric secretion in some cases but treatment was generally of only symptomatic value suggesting irreversible damage.

In the spring of 1943 we prepared an infusion of rice bran or millings hoping to extract elements of the B complex. This infusion was then used in place of the water in our yeast culture. Unfortunately, soon after, the issue of sugar was stopped, the general ration cut to its previous low level and we were no longer allowed to have the rice bran. It was impossible to continue either the yeast culture or the preparation of the infusion.

With the decrease in the ration and the exhaustion of the Red Cross foods cases of pellagra began to recur and we found the pure vitamin preparation to have much less effect in cases of comparable severity. The stomatitis and dermatitis would improve slightly as long as a man was maintained on a high nicotinic acid intake, only to relapse promptly when the vitamin was withdrawn. Our supply of nicotinic acid dwindled rapidly and we were again forced to treat the more severe cases at the expense of those less severe.

The incidence and severity of the disease had again reached serious proportions by the fall of 1943. Fortunately, in December, 1943, another Red Cross shipment of food and medicine arrived. A quantity of nicotinic acid tablets and multivitamin capsules containing 10 mg of nicotinic acid was included. Every man received 1 multivitamin capsule daily and all those with signs of pellagra were given nicotinic acid in amounts similar to those used the year before.

Again we noted the increased effect of pure vitamin preparations when the diet was more nearly adequate in protein and B complex content. By conserving and using the vitamin preparations sparingly we were able to continue distribution of 1 multivitamin daily until the time of our release by the American Forces in January, 1945.

Early in 1944 the ration was cut to less than 1200 cal. and during the year the ration was gradually cut more. By June

those doing hard physical work were being issued less than 1000 cal. and the others less than 800 cal. per day. The diet was augmented some by individual gardens.

Throughout the year chronic mild pellagra continued to affect 25% to 50% of the prisoners despite daily administration of 1 multivitamin capsule. Relapses often followed diseases like dengue, malaria, or dysentery in which fever, anorexia and vomiting were prominent symptoms. Remissions followed increase in multivitamin intake and nicotinic acid in doses of 25 to 100 mg per day. Small quantities of fruit, cocoanut, mongo beans, or carabao meat secured from the Filipinos were given as an extra ration to the worst patients.

We observed that once a man had developed pellagra he was much more susceptible to the disease and more resistant to treatment under the same dietary conditions than the man who somehow had escaped the severe form of the disease in 1942.

The morbidity from pellagra in 1944 was less serious than we had anticipated in view of the very inadequate diet. We concluded that the daily multivitamin, although not preventing the appearance of pellagra, tended to prevent the severe fulminating form seen in 1942.

SUMMARY

1. Pellagra was observed in American troops living for 3 years on a deficient diet in Japanese Prison Camps in the Philippine Islands.

2. Men developed pellagra while on a diet low in calories, high in carbohydrates and lacking in animal proteins, fresh fruits, and vegetables.

3. Pellagra appeared in epidemic proportions after 6 months on a deficient diet.

4. The signs and symptoms that were observed are described. The clear separation of certain dermal and neurological lesions according to specific vitamin lack was difficult.

5. Processed foods, a crude yeast culture, and pure vitamin preparations were effective in controlling pellagra. Pure vitamin preparations were more effective when the diet was more adequate.

6. The daily multivitamins utilized during 1944 reduced the incidence of the severe pellagra seen in 1942 and 1943.

PHOTOSENSITIZED OXIDATION OF ASCORBIC ACID IN URINE AND BLOOD SERUM

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Martini reported in 1934 the photodynamic action of flavins on ascorbic acid solutions. Kon and Watson ('36) and Kon ('37) published data showing that the ascorbic acid in both raw and pasteurized milk was reversibly oxidized on exposure to light, that short wave lengths of visible light were responsible, that the biologically active product formed underwent further decomposition without the agency of light and that oxygen was required for the reaction. Hand, Guthrie and Sharp ('38) stated that since dissolved oxygen and ascorbic acid solutions are colorless and do not absorb light, another substance must be involved in this photosensitized reaction. They showed the substance to be riboflavin and claimed it to be the sole agent responsible for the oxidation of ascorbic acid in milk in a pH range from 5 to 9.6. Hand and Greisen ('42) demonstrated that riboflavin and light oxidized ascorbic acid in solutions at a range in pH of 3.9 to 7.7 and compared this oxidation with that caused by copper and by cucumber oxidase.

More recently data have accumulated showing the loss of riboflavin in milk on exposure to visible light (Peterson, Haig and Shaw, '44; Ziegler, '44; Stamberg and Theophilus, '44). Holmes and Jones ('45) have called attention to the simultaneous loss of reduced ascorbic acid. The effect of various lighting conditions on riboflavin solutions has been investigated by DeMerre and Brown ('44).

During a study of human requirements of ascorbic acid in this laboratory it was observed that reduced ascorbic acid in urine, preserved at pH 2 to 3, also was destroyed on exposure to light. Even on low riboflavin intakes some excretion of riboflavin occurs (Sebrell, Butler, Wooley and Isbell, '41; Hagedorn, Kyhos, Germek and Sevringhaus, '45). Riboflavin and possibly other fluorescent constituents of urine can cause this photosensitized oxidation of the reduced ascorbic acid. Since blood serum contains the components necessary for this photochemical activity, the effect of light on it has been tested also.

Data are presented to show that a photosensitized oxidation of reduced ascorbic acid occurs in urine preserved at pH 2 to 3, that after the oxidation dehydroascorbic acid is present and that the reaction depends upon the presence of dissolved oxygen. Riboflavin is an active agent in this change since increasing amounts accelerate the reaction. It is shown, too, that the concentration of reduced ascorbic acid in blood serum is decreased on exposure of the serum to light.

EXPERIMENTAL

Urine

Reduced ascorbic acid in urine was determined by the method of Evelyn, Malloy and Rosen ('38), and the total ascorbic acid by that of Roe and Kuether ('43). At the end of each exposure period the samples were stored in the dark and all analyses were made at the end of the total exposure time. The urine samples were collected directly after taking ascorbic acid in order to produce a urine of sufficiently high ascorbic acid content to show the progressive destruction during a 3-hour period. The samples were brought to pH 2.5 by the addition of glacial acetic acid. An ascorbic acid intake of 100 mg per day by a normal individual results in a 24-hour urine excretion of approximately 3 mg per 100 ml.

The effects of wave length and intensity of light were not studied. However, in order to have a linear relationship be-

tween loss of reduced ascorbic acid and time a constant light source was necessary. A 300-watt tungsten filament bulb furnished an intense light but evidently not of optimum wave length, since less intense skyshine caused a more rapid destruction (table 1). The removal of oxygen by dry ice checked the destruction (table 2). The effect of temperature was not investigated.

TABLE 1

Photochemical oxidation of ascorbic acid in urine.[1]

TIME OF EXPOSURE	CONSTANT LIGHT [2] 500 FOOT-CANDLES 33–35°C.		SKYSHINE,[3] 150 INCREASING TO 300 FOOT-CANDLES 28–29°C.	
Min.	Reduced ascorbic acid	Total ascorbic acid	Reduced ascorbic acid	Total ascorbic acid
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
0	7.85	7.85	7.85	7.85
30	7.45	7.55	7.05	8.00
60	6.95	7.70	6.50	8.00
90	6.55	7.65	5.55	8.25
120	6.05	7.65	4.80	8.15
150	5.60	7.65	3.90	8.15
180	5.25	7.55	2.55	7.85

[1] Urine, pH 2.5, exposed in 25 ml quantities.

[2] Constant light furnished by 300-watt tungsten filament bulb at 15 inches, 500 ± 50 foot-candles, depending on angle of incidence.

[3] Skyshine, exposure in an open north window, tree-shaded. Light measured by Weston meter.

TABLE 2

Effect of oxygen removal (dry ice added for 45 minutes prior to exposure) on the photochemical oxidation of ascorbic acid in urine.[1]

TIME OF EXPOSURE	CONSTANT LIGHT [1] 500 FOOT-CANDLES 35–36°C.		SKYSHINE,[1] FLUCTUATING BETWEEN 100 AND 140 FOOT-CANDLES 28–29°C.	
Min.	Reduced ascorbic acid	Total ascorbic acid	Reduced ascorbic acid	Total ascorbic acid
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
0	9.60	9.90	9.60	9.90
60	9.50	9.30	9.35	9.65
120	9.40	9.80	9.05	10.0
180	9.45	9.20	8.75	9.65

[1] See footnotes, table 1.

Although increasing amounts of riboflavin have been shown to accelerate the destruction of reduced ascorbic acid (table 3), riboflavin is not the only factor in urine which may so act. It is a normal constituent and is typical of other fluorescent and dye substances which may accept hydrogen from reduced ascorbic acid.

TABLE 3

Effect of added riboflavin on the photochemical oxidation of ascorbic acid in urine.[1]

RIBOFLAVIN ADDED	CONSTANT LIGHT,[1] 60 MIN. EXPOSURE	
	Reduced ascorbic acid	Total ascorbic acid
mg	*mg/100 ml*	*mg/100 ml*
0	5.70	6.70
0.08	5.20	6.75
0.16	4.95	6.95
0.32	4.60	6.90
0.64	4.10	6.95

[1] See footnotes, table 1.

Blood

Table 4 presents data illustrating the effect of light on samples of the same human blood serum, clear and hemolyzed, exposed in centrifuge tubes in the 0.1 ml quantities used for analysis. At the end of the exposure periods the tubes were stored in the dark. Protein precipitation and analysis for reduced ascorbic acid by the Mindlin and Butler ('38) micromethod followed at the end of the total exposure time. In addition to the exposure to the constant light used, samples were also exposed to a white fluorescent 15-watt light, at 12 inches.

While these data are not analogous to those given for urine since there are no total ascorbic acid values, they show the loss of reduced ascorbic acid in blood serum on exposure to light and emphasize the necessity in this analysis for immediate precipitation of protein and removal with it of the effective catalyzing agents.

The lowered reduced ascorbic acid content of hemolyzed blood serum is well recognized. These data suggest that this loss has occurred sometime prior to exposure and the rate of loss in these slightly hemolyzed samples thereafter is approximately the same as for the clear serum. The degree of hemolysis may alter this rate of change. There have been indications throughout the work that the red color liberated in severe hemolysis protects the reduced ascorbic acid present from the photosensitized oxidation.

The pH of blood serum makes the alteration of reduced ascorbic acid in vitro in this medium more complicated than

TABLE 4

Reduced ascorbic acid of human blood serum exposed to constant light and white light.

TIME	CLEAR SERUM		HEMOLIZED SERUM	
Min.	Reduced ascorbic acid		Reduced ascorbic acid	
	Constant light [1]	White light [2]	Constant light	White light
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
0	1.12	1.12	0.86	0.82
60	0.73	...	0.58	...
120	0.41	0.40	0.25	0.24
180	0.14	...	0.0	...
240	0.13	0.20	0.0	0.0

[1] See footnote 2, table 1.

[2] 15-watt Mazda fluorescent white light at 12 inches.

that in the acidified urine. There is in all probability a secondary loss, due to the pH, superimposed on the light-catalyzed reaction. The data do demonstrate, however, an oxidation which is stopped when light is removed.

In the course of the blood work it was found that the light from a 15-watt white fluorescent light tube at 12 inches was as effective in oxidizing reduced ascorbic acid as was that from the 300-watt tungsten filament bulb used as a constant light source. The white light and daylight lamps radiate a greater proportion of the light effective in this light-

sensitized reaction (ultraviolet and short visible rays) than does the more intense tungsten filament bulb used as a source of constant light (Barnes, Forsythe and Karash, '39).

The above findings indicate that during the routine preparation of a sample for determination of reduced ascorbic acid by indophenol methods, a significant loss may occur both in urine and blood serum. It is important, therefore, to guard against exposure to light samples which are being analyzed for reduced ascorbic acid. The similarity of conditions producing the photochemical destruction of riboflavin and reduced ascorbic acid suggests an interdependence of these two compounds leading to their mutual alteration and the need for like precautions in the analysis of each when the other is present.

SUMMARY

1. The photosensitized oxidation of reduced ascorbic acid in urine preserved at pH 2 to 3 has been shown to take place.

2. The ascorbic acid is present in the urine after oxidation as dehydroascorbic acid.

3. The reaction depends upon the presence of dissolved oxygen.

4. The reaction is accelerated by increasing amounts of riboflavin.

5. A loss of reduced ascorbic acid occurs in blood serum exposed to light.

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THE INFLUENCE OF RIBOFLAVIN CONSUMPTION ON ITS CONCENTRATION IN HENS' EGGS

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TWO FIGURES

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This paper is a report of a series of experiments designed to investigate the efficiency with which hens transfer riboflavin from their feed to the eggs and the influence of the level of the dietary riboflavin on this efficiency. Data indicating the effects of breed and variety, variations between individuals and with the same individual and the variation over a period of several months of experimental feeding are included. The investigation was undertaken to determine the practicability of introducing additional riboflavin into the human dietary through the medium of eggs laid by hens fed the vitamin.

The level of riboflavin in the feed has been shown to play an important part in the hatchability of the eggs produced. Lepkovsky, Taylor, Jukes and Almquist ('38), Hunt, Winter and Bethke ('39) and Engel, Phillips and Halpin ('40) have reported a direct variation of the riboflavin content of the eggs with that of the ration of the laying birds. Norris and Bauernfeind ('40) found that the riboflavin concentration of the eggs could be increased to a maximum level beyond which increases in dietary riboflavin had no effect. This level was 800 to 1000 μg of riboflavin per 100 gm of ration. No reports

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have appeared to date of experiments on the balance between riboflavin intake and output.

Sherman and Lanford ('38) reported that, on the average, a whole egg contained 65–70 Sherman-Bourquin units. They also state that one Sherman-Bourquin unit was equivalent to 3–5 μg. From the result of Hunt, Winter and Bethke ('39) it would appear that the riboflavin content of eggs laid by hens fed the usual type of ration was about 200 to 250 μg. The maximum values of Norris and Bauerfeind ('40) calculated for the whole egg were approximately 170 μg. Peterson, Dearstyne, Comstock and Weldon ('45), using a fluorometric determination, found great differences between birds in the riboflavin content of their eggs. The average value for whole eggs was 3.2 μg per gm (about 163 μg per egg). The most recent compilation of the U. S. Department of Agriculture and the National Research Council ('45) gives a riboflavin value of 3.4 μg per gm of fresh whole egg (about 173 μg per egg).

METHODS

The ration samples were analyzed by the microbiological procedure of Snell and Strong ('39) with a modified extraction method that had been previously used for the assay of cereals (Jackson, Doherty and Malone, '43). Occasional samples of the rations containing the higher levels of riboflavin were also assayed by a suitable fluorometric procedure with satisfactory checks.

The eggs were assayed by a modified Hodson and Norris ('39) type of fluorometric procedure.[2] All the eggs comprising a sample were broken out and mixed in a Waring blender. A weighed sample of the melange was mixed with two parts of water to give an even suspension that could be pipetted readily. Ten ml of this suspension were mixed with 10 ml of water and 20 ml of 0.2 N HCl in a 125 ml flask. After steaming for 15 minutes and autoclaving for another 15 min-

[2] This method was developed by D. M. Young, Department of Animal Nutrition, Ontario Agricultural College, with the cooperation of the Ontario Research Foundation, Toronto. The technique was modified in some minor respects.

utes at 15 pounds pressure, the flask was cooled and the contents adjusted to pH 5.0 with 0.5 N NaOH, using a glass electrode pH meter. The mixture was diluted to 50 ml with water and filtered. The filtrate was carefully adjusted to pH 6.5, using 40% NaOH. A 15 ml aliquot of this water extract was diluted to 50 ml with acetone and kept over night at 4°C. It was filtered while cold through a Whatman no. 3 filter paper. A perfectly clear filtrate was obtained. Two 15 ml aliquots of this filtrate were pipetted into each of two test tubes; 0.10 ml of a standard riboflavin solution containing 20 μg per ml was added to one tube and mixed. The total fluorescence of each solution was then determined in a Pfaltz and Bauer fluorometer. The difference between the readings, corrected for the small dilution caused by addition of the standard, gave the fluorescence equivalent to 2 μg of riboflavin. The blank was obtained by adding 0.2 ml of fresh, ice-cold 5% $Na_2S_2O_4$ solution in 5% $NaHCO_3$ to the cuvette. The reading was taken immediately after mixing, before any oxidation of the riboflavin by the air could take place. Recoveries of added riboflavin were from 95 to 99%. Checks were consistently within $\pm$ 3%, and the results obtained by this method agreed closely with those obtained by a more elaborate procedure, a modification of the Najjar ('41) procedure. All riboflavin assays were conducted in a dark room under red illumination.

Experiment no. 1

This experiment was designed to study the riboflavin content of eggs laid by birds maintained in the conventional type of house and fed practical rations supplying different levels of riboflavin. In addition, since Bird and Marvel ('43) had reported that ingestion of feces eliminated the effect of low riboflavin rations on hatchability, it was considered advisable to include birds kept in batteries, to eliminate coprophagy insofar as possible. Accordingly, Barred Plymouth Rock pullets were grouped in six pens and in two

groups of individual hen batteries. The number of birds in each pen varied during the course of the experiment, due to mortality, but did not drop below twenty-five at any time.

Four rations, numbered 1 to 4, were designed to contain, respectively, 800 μg, 1200 μg, 1600 μg and 2000 μg of riboflavin per pound. A basal mash of the following composition, ground yellow corn 10.0, rolled oats 12.0, ground oats 10.0, wheat bran 9.0, wheat shorts 4.0, dehydrated alfalfa meal 4.0, dehydrated cereal grass 2.0, meat meal 5.0, fish meal 2.5, soy bean oil meal 8.0, iodized salt 1.0, oyster shell 2.5, bone

TABLE 1

Composition of rations (lbs.).

INGREDIENT	RATION			
	1	2	3	4
Basal mixture	71.00	71.00	71.00	71.00
Casein (vitamin-free)	1.75	....	....	....
Buttermilk powder	....	5.00	5.00	5.00
Soybean oil meal	....	1.25	1.25	1.25
Barley	15.25	10.75	10.75	10.75
Wheat	12.00	11.50	8.00	4.25
Riboflavin pre mix [1]	0.0	0.50	4.00	7.75

[1] This was a mixture of 100 pounds of ground wheat and 2.0 gm of synthetic, crystalline riboflavin. The riboflavin was furnished by Merck and Company, Limited, Montreal.

meal 0.5, fish liver oil (3500 A, 400 D) 0.5, and anhydrous manganous sulphate 0.025 pounds, was used. The composition of the rations is shown in table 1. Vitamin-free casein was used to supply milk protein in ration no. 1, in an amount equivalent to that supplied by buttermilk powder in the other rations. The crude protein content of these rations was equalized at approximately 15%. The common practice of feeding scratch grain with laying mashes of this type was not feasible in this experiment as consumption of uniform proportions of mash and grain was desired. Therefore, ground

scratch grain was mixed with the mash in the proportion [3] of 1 of grain to 1.3 of mash in all groups.

Pen no. 1 and Battery no. 1 received ration no. 1, pen no. 2 received ration no. 2, pen no. 3 and Battery no. 2 received ration no. 3 and pen no. 4 received ration no. 4. For comparative purposes two additional groups were included. Pen no. 5 received a commercial laying mash and no. 6 received a commercial hatching ration. Scratch grain was incorporated with both rations as above. Oyster shell and grit were supplied to all groups ad libitum, and the amounts consumed were recorded.

The riboflavin content of each of the complete rations was determined by assays of samples of each new batch as prepared at about 2-week intervals. Ration no. 1 averaged 964 μg per pound over the course of the experiment, as determined by assay. Ration no. 2 assayed 1350 μg per pound; Ration no. 3 assayed 1669 μg per pound, while Ration no. 4 assayed 2301 μg per pound. The commercial laying mash and the commercial hatching mash, each mixed with ground grains in the proportion 1.3:1, averaged 980 and 1390 μg per pound, respectively. The recommended allowances set forth by the National Research Council ('44) are not less than 800 μg per pound for egg production and 1300 μg per pound for hatching rations. The actual levels of riboflavin fed, as determined by assay, are shown graphically in figure 1.

An initial sample of eggs was collected at the start of the experiment and thereafter at weekly intervals for 4 weeks. At this time the riboflavin content of the eggs appeared to have become reasonably constant for each group. Samples were then collected at intervals of about 4 weeks. Each sample consisted of about forty eggs and was representative of all eggs laid over a 3-day period. The concentration of riboflavin found in these samples, expressed as μg per egg is illustrated in figure 1.

[3] This proportion was determined by examination of the records of actual relative consumption for some years of mash and grain by birds of similar breeding fed on the same type of rations.

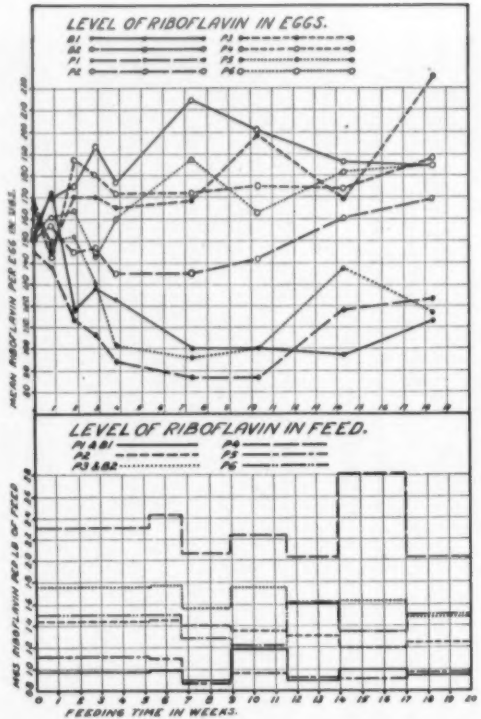

Fig. 1 The relation between the concentration of riboflavin in the chicken feed and the concentration found in the eggs.

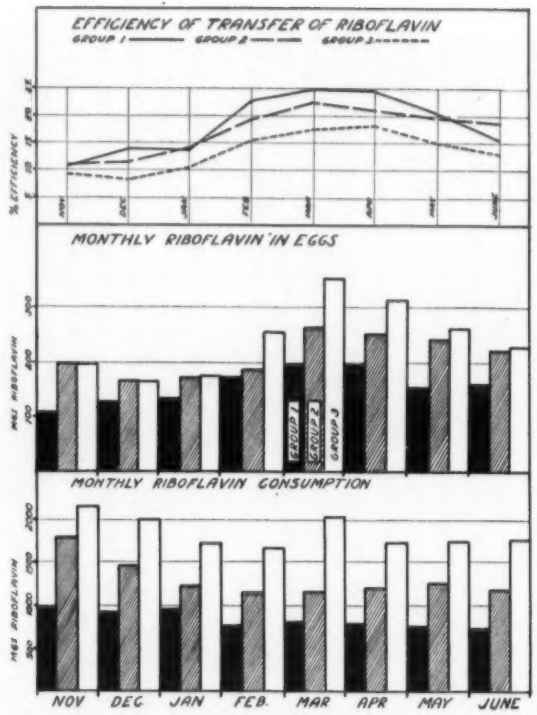

Fig. 2 The variations in riboflavin consumption, riboflavin recovered in the eggs, and efficiency of transfer from the feed to the eggs over an 8-month period.

An initial rapid adjustment of the riboflavin level in the eggs took place and was reasonably complete in a period of 3 to 4 weeks after beginning the experimental rations. After this initial adjustment Battery 2, and Pens 3, 4 and 6 formed a broad group with fluctuating values ranging from 160 to 225 μg per egg, averaging about 185 μg per egg. Battery 1, Pen 1 and Pen 5 formed a lower group, averaging about 100 μg per egg. Pen 2 was midway between these groups.

The proportion of dietary riboflavin recovered in the eggs was calculated for each pen and battery for the period from the fourth to the eighteenth week on the experimental rations. The total riboflavin consumed was calculated from the total feed consumption and the average level of riboflavin in the feed. The total riboflavin recovered was calculated from the total egg production and the average amount of riboflavin per egg for the period. Table 2 shows the results of these calculations.

TABLE 2

The riboflavin balance during the period from the fourth to the eighteenth week of experimental rations.[1]

PEN	TOTAL EGG PRODUCTION	AV. WT. EGG CONTENTS	TOTAL FEED CONSUMPTION	AVERAGE RIBOFLAVIN		TOTAL RIBOFLAVIN		RIBOFLAVIN FROM FEEDS RECOVERED IN EGGS
				In feed[2]	In eggs	Intake in feed	Output in eggs	
		gm.	*lbs.*	*μg/lb.*	*μg/egg*	*mg*	*mg*	%
1	1300	51.5	734	985	105	723	136.5	18.9
2	1509	51.1	704	1310	148	922	223.5	24.2
3	1649	52.9	838	1600	178	1340	294.0	21.9
4	1162	50.8	625	2230	178	1395	207.0	14.8
5	1129	50.6	847	940	112	796	126.5	15.9
6	1808	52.1	983	1380	179	1360	324.0	23.8
Battery 1	1443	51.8	569	985	103	560	149.0	26.6
Battery 2	1429	54.4	593	1600	197	950	281.5	29.6

[1] March 15 to June 15.

[2] Note that these average values are for the feed consumed during the fourth to eighteenth week only, while figures reported in the text are for the entire experiment.

There was a maximum percentage recovery of the riboflavin in the eggs from birds maintained in pens when the riboflavin content of the feed was about 1300 μg per pound. It is probable that the hen has a definite requirement for body maintenance and normal activity. Hence at lower concentrations of intake, a higher proportion of this intake will be required for maintenance and activity. However, there appears to be a limit to the ability of the bird to efficiently transfer riboflavin to the egg when the available surplus increases. This finding corroborates the report of Norris and Bauernfeind ('40) that there is a maximum level of intake beyond which no increase in the riboflavin content of the egg is achieved. However, the Cornell workers reported this dietary level as 3600 to 4500 μg per pound of ration, whereas this level in our work was about 1400–1600 μg in the case of the birds housed in the conventional manner. An average concentration of 179 μg of riboflavin per egg was the maximum obtainable even when the dietary level was raised to 2230 μg per pound.

The highest recoveries of riboflavin were made by the birds maintained in batteries. This greater recovery in Battery 1, at the level of 985 μg of riboflavin per pound of feed, was entirely due to the production of a greater number of eggs per pound of feed consumed, as the concentration of riboflavin was the same in the eggs from Pen 1 and Battery 1. Raising the level of dietary riboflavin to 1600 μg per pound (Pen 3 and Battery 3) resulted in a greater increase of the level of riboflavin in the eggs from the battery than from the corresponding pen. This greater recovery for Battery 2 resulted from both a greater egg production per pound of feed consumed and a higher concentration per egg. The activity of birds in batteries is less than that of birds housed in pens and this may be responsible, in part at least, for the more efficient egg production per pound of feed consumed.

Since the riboflavin content of the eggs laid by the birds in batteries was as high as, or higher than, those laid by birds fed the same ration but housed in pens, it would appear

that coprophagy does not increase the riboflavin content of the eggs laid.

Experiment no. 2

The opportunity was taken during the foregoing experiment to investigate the variation in performance of individual hens. About twelve hens were selected from each of Pens 1, 2 and 3 and five collections of individual egg samples were made. Each sample consisted of all the eggs laid by one bird for a week. The maximum, minimum and average riboflavin contents of the eggs of these birds are listed in table 3. A considerable range was observed for each bird. This range in riboflavin content of eggs laid by any individual bird is much wider than that observed in other studies carried out over shorter periods at the Ontario Agricultural College.[4] There is also evidence of variation between individuals in ability to transfer the vitamin from the feed to the egg as reported by Peterson et al. ('45).

Experiment no. 3

After completion of the foregoing experiments and examination of the results, it was decided to repeat the study, expanding its scope by extending it over a longer period of time and elevating the upper level of riboflavin in the feed. Accordingly, 225 Barred Plymouth Rock pullets were divided into three groups, each group consisting of three pens of twenty-five birds each. The pens were kept up to strength throughout the study by replacement of any losses by birds of similar breeding. Eggs laid by these replacements were not used for assay purposes until the bird had been on the ration for at least 4 weeks. The birds were fed an "all mash" ration made up with the same formula as that previously used. The mean value of the assays of all samples of feed for Group 1 for the period of study was 1185 μg per pound of feed, the mean for Group 2 was 1870 μg per pound and that for

[4] Unpublished data, Department of Animal Nutrition and Department of Poultry Husbandry.

TABLE 3

Riboflavin content of egg samples from individual hens. Experiment no. 2.

PEN NO. 1 RIBOFLAVIN — 985 μG/LB.[1]			PEN NO. 2 RIBOFLAVIN — 1310 μG/LB.[1]			PEN NO. 3 RIBOFLAVIN — 1600 μG/LB.[1]		
Bird no.	Riboflavin/egg		Bird no.	Riboflavin/egg		Bird no.	Riboflavin/egg	
	Range	Average		Range	Average		Range	Average
	μg	μg		μg	μg		μg	μg
903	32–108	68	943	84–163	135	985		123
904	76–143	110	944	150–182	166	986	143–176	160
906	85–108	99	946	94–152	118	987	183–234	214
910	88–129	114	947	127–162	149	989	131–155	142
911	90–164	124	948	99–168	135	994	179–235	192
912	64–151	106	949	153–220	193	993	133–191	157
915	74–119	92	951	97–171	127	996	170–225	192
916	74–133	101	952	101–195	149	997	139–171	149
917		92	954	133–200	161	998	100–163	131
919	60–91	75	958	101–185	149	999	119–133	128
920	82–167	107	960	110–245	178	1401	121–171	149
921	91–119	109	961	107–145	124	1404	138–194	173
923	62–101	83	962	81–135	115	1402	150–171	161
924	115–135	125	967		114	1405	127–178	147
929	83–106	95	968		80	1410		212

[1] Average riboflavin content of the feed after the initial adjustment period.

Group 3 was 2560 μg per pound. The study was maintained for a period of 8 months, from November to June inclusive. A large representative sample of eggs from each group was collected over several days and assayed by the fluorometric procedure. The results are illustrated graphically in figure 2.

The monthly riboflavin consumption for each group was calculated from the feed consumed each month and the riboflavin assays of the batches of feed used during the month. The monthly output in the eggs for each group was calculated from the total egg production for each month and the riboflavin assay of the egg sample for that month. The efficiency of transfer of riboflavin from the feed to the eggs for each month was calculated from the riboflavin recovered in the eggs each month and the riboflavin consumption.

The main point of interest revealed by this study was the relatively large increase in total riboflavin output in the eggs during February, March, April and May. This increase was due largely to an increase in total egg production during these months, but the concentration of riboflavin in the eggs followed the same general variation in a lesser degree. This cycle was reflected in the efficiency curves.

The larger amount of dietary riboflavin supplied to Group 2 as compared to Group 1 resulted in an approximately proportional increase in the riboflavin output in the eggs for each month, with the exception of February. On the other hand, the increased dietary riboflavin consumed by Group 3 compared to Group 2 did not result in any significant increase in riboflavin output in the eggs during November, December, January and June but it did produce a definite increase in riboflavin output in the eggs in February, March, April and to a lesser extent, in May. This increase was the result of a higher concentration of riboflavin in the eggs and not the result of a gain in egg production by Group 3 over Group 2. Hence it appears that during the period of maximum egg production there was also an elevation of the upper level of dietary riboflavin that will influence the concentration of riboflavin in the eggs. The upper effective level of dietary ribo-

flavin was ordinarily about 1600 μg per pound of feed but it was more than 1870 μg per pound of feed during the period of maximum egg production and may have reached as high a level as 2500 μg per pound or even higher. It is possible that higher levels of riboflavin, had they been used, would have been found to raise the concentration of the vitamin in the egg still more. This finding, which was not obvious in the first experiment, probably explains, in part at least, the discrepancy between our original observation and that of the Cornell workers with regard to the maximum effective level.

Experiment no. 4

As a preliminary investigation, egg samples were also collected from a number of breeds and varieties of poultry maintained in pens and fed on the commercial hatching ration used in the first experiment. This ration had an average

TABLE 4

Riboflavin in eggs from different breeds and varieties of poultry fed same ration.

BREED AND VARIETY	AVERAGE WEIGHT EGG CONTENTS	RIBOFLAVIN	RIBOFLAVIN
	gm	*μg/gm*	*μg/egg*
Barred Plymouth Rock	52.5	3.21	168
White Plymouth Rock	48.6	3.03	147
New Hampshires	53.6	3.15	168
White Wyandottes	51.2	3.45	177
White Chanteclers	54.0	3.41	184
New Ontario [1]	47.7	2.91	139
Jersey Black Giants	51.6	3.38	174
Light Sussex	53.0	3.68	195
Light Sussex (yellow skinned) [2]	52.6	3.36	177
Black Langshans	57.0	3.71	211
White Leghorns	54.2	3.65	198
Brown Leghorns	47.3	3.43	162

[1] This breed was developed at the Ontario Agricultural College in 1910. The foundation breeds were Light Sussex, White Laced Cornish and Light Red Brahmas.

[2] This was a strain of Light Sussex selected for yellow skin. The Light Sussex breed normally has white skin.

riboflavin content of 1380 μg per pound but feed consumption records were not available for the particular period of the test. All egg samples were collected at the same time and consisted of at least thirty eggs per sample with the exception of the Black Langshans' sample which consisted of only seven eggs. The assays are shown in table 4.

The riboflavin values of the eggs were reasonably constant, although the suggestion of a breed and/or variety difference does exist. However, it is unlikely that any true breed or variety difference that might be shown by a more complete investigation of these breeds and varieties would prove to be of practical importance.

DISCUSSION

The primary objective of this investigation, as previously stated, was to discover the feasibility of supplying riboflavin in the human diet by feeding the vitamin to hens and recovering it in the eggs. Some calculations based on the data given in table 2 show that such an addition would be comparatively efficient if the proper concentrations of riboflavin in the feed were observed. The usual type of laying ration contains about 1000 μg of riboflavin per pound and was represented by Pens 1 and 5 in the first experiment. The eggs from these pens averaged about 110 μg of riboflavin per egg. This was raised to 148 μg per egg in Pen 2 and to 179 μg per egg in Pen 6 by raising the riboflavin in the feed to 1310 and 1380 μg per pound, respectively, that is, to the usual hatching ration level. A consideration of Pens 1 and 2 in the first experiment shows that each pound of feed in Pen 1 contained 985 μg of riboflavin and 18.9% or 186 μg of this was recovered in the eggs. Each pound of feed in Pen 2 contained 1310 μg of riboflavin and 24.2% or 317 μg of this was recovered in the egg. Hence, from an addition of 325 μg of riboflavin per pound of feed, 131 μg were recovered in the eggs. Thus 40.3% of the extra riboflavin required to raise the dietary level from 985 to

1310 μg per pound was recovered. A similar calculation with the data for Pens 5 and 6 shows that 40.5% of the extra riboflavin was recovered.

This might prove to be an economical method of recovering riboflavin from such sources as buttermilk or skim milk powder, dried whey, liver meal and so forth. These products are not ordinarily consumed by humans.

SUMMARY

Barred Plymouth Rock pullets were divided into groups and supplied with rations containing different concentrations of riboflavin. The riboflavin in the feed and the eggs was determined by assay of representative samples and the total riboflavin consumed and that recovered in the eggs was calculated. The percentage recovery of the dietary riboflavin was calculated.

A maximum recovery in the eggs laid of about 24.2% was found at a dietary level of 1300 μg per pound of feed for birds in pens. When they were kept in batteries the recovery increased to 29.6% at 1600 μg per pound of feed.

The highest level of dietary riboflavin that would affect the concentration of riboflavin in the eggs was ordinarily about 1400–1600 μg per pound of feed, but this level was found to be considerably higher during the period of high egg production. The percentage recovery in the eggs was also raised during this period.

There was a higher recovery of riboflavin in the eggs from birds maintained in batteries than from those in ordinary pens.

About 40% of the extra riboflavin required to raise the concentration in the feed from 1000 μg to 1350 μg per pound was recovered in the eggs.

There was some variation in the concentration of riboflavin in the eggs from various breeds and varieties on the same ration. There was also variation between individual hens on the same ration.

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